VOL. XXXIX SEPTEMBER, 1919 No. 2

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

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PUBLISHED MONTHLY
AT 3419-21 GREENMOUNT AVENUE, BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
FOR THE JOURNAL OF BIOLOGICAL CHEMISTRY, INC.

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Entered as second-class matter August 1, 1911, at the Post Office at Baltimore, Md., under the act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in Section 1103, Act of October 3, 1917. Authorized on June 29, 1918.

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NOTE ON THE IMPORTANCE OF ACCURATE AND QUANTITATIVE MEASUREMENTS IN EXPERIMENTAL WORK ON NUTRITION AND ACCESSORY FOOD FACTORS.

By HARRIETTE CHICK and E. MARGARET HUME.

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(Received for publication, July 7, 1919.)

The object of this note is to submit a criticism upon much of the experimental work recently published on the subject of accessory food factors, and upon the conclusions drawn as to the value of various foodstuffs in this respect.

Since the general conception of accessory food factors (or vitamines) was brought forward as the result of the work on the antiberi-beri (or antineuritic factor) by Eijkman, Grijns, Fraser, Stanton, Funk, and others; on the antiscorbutic factor by Holst and his coworkers; on the "growth factors" by McCollum and his colleagues, a constant succession of researches has been published in which attempts have been made to assign values to various foodstuffs as antineuritic, as antiscorbutic, or as growth-promoting materials respectively, and further to determine whether these values are lessened or destroyed by heating, drying, or other methods of preservation. In most of this work one fundamental consideration, namely that of *quantity*, has been to a large extent overlooked in the methods adopted for experiment, and the conclusions drawn have been untrustworthy to a corresponding degree.

In comparing the value of a series of foodstuffs as regards their value in content of some accessory food factors, it is obvious that the first step necessary is to determine in each case the minimum daily dose which will maintain health in the experimental animal and to institute comparison between these amounts. These daily doses should be as large as can be tolerated before it is safe to deny

to a foodstuff any value in respect of the particular accessory factor under consideration.

Similarly, in investigating the effect of heat, drying, or any other method of preservation upon the accessory factors contained in any foodstuff, it is necessary to determine the minimum daily dose (1) of the original untreated material and (2) of the same material after heating, drying, etc., which will protect the same experimental animal under similar conditions of experiment. Then only is it possible to state to what extent, if any, the foodstuff has suffered deterioration as the result of the treatment.

Neglect of this simple and fundamental, but admittedly tedious procedure is evident in many papers recently published upon the influence of various treatment upon the antineuritic and antiscorbutic and other properties of certain foodstuffs, and the results obtained are in consequence vague, and may even be erroneous. It is clear, for example, that if a foodstuff is rich in any particular accessory factor and is fed to an animal in large excess of the amount actually required, any destruction of the factor that may take place during heating cannot be assessed and may even remain undetected if the heated material is fed in similar large amount. It is absolutely necessary to determine the minimum required in both cases, and to institute a comparison between these two results before any satisfactory conclusion on this point can be drawn.

We have selected a recent paper on the "Influence of high temperatures and dilute alkalies on the antineuritic properties of foods" by Daniels and McClurg (1919) as affording a particularly well marked illustration of this point, but other examples may be found in the same journal during the last 2 or 3 years. These authors studied the content of antineuritic factor ("water-soluble growth factor") in navy beans, soy beans, and cabbage after exposure to heat, and after treatment with alkali. This was done by means of nutritional experiments with rats, observing the rate of growth when these substances were added to a basal diet consisting of salts, butter fat, casein, lard, and corn-starch. In the case of the beans the quantity given amounted to about half the dry weight of the total diet; in the case of cabbage, 250 gm. (gross weight) were added to 100 gm. of the basal diet. The intake of food was not measured. The expressed aim of the authors was

"to determine the effect on the antineuritic vitamine in vegetables prepared in accordance with the usual household and canning processes;" the cabbage and beans were therefore boiled with sodium bicarbonate in some experiments and heated to 120°C. for periods of time ranging from 15 to 40 minutes in others. No determination was made of the minimum daily ration of raw beans or of raw cabbage which will promote satisfactory growth in the rat when supplied as the sole source of antineuritic vitamine; there is no record of food offered or residues measured, consequently none of the total food intake.

In all cases the animals grew and thrived well. The proportion of beans and cabbage in the diet used by these authors is very high. Such a diet would, it may safely be affirmed, contain so large an excess of the antineuritic vitamine that the total amount could well be halved, quartered, or even further reduced without in any way interfering with the animals' well being; and yet the authors feel justified in saying (p. 208) that the experiments "indicate that neither high temperatures nor dilute alkalies at the boiling temperature are very detrimental to the antineuritic vitamines in foods." The final conclusion reached (p. 212) is that "the note of warning sounded by Chick and Hume to the effect that grave danger may attend the use of large amounts of tinned foods, we believe, is unfounded, at least from the standpoint of the antineuritic vitamine content of the food."

The note of warning referred to above (Chick and Hume, 1917–19) was sounded as the result of experiments in which the antineuritic value of (1) yeast and (2) wheat embryo was determined before and after heating to temperatures ranging from 100 to 124°C. for periods of time varying from 1 to 2 hours. Accurate determination was made of the minmium curative dose required to cure pigeons suffering from acute polyneuritis brought on by an exclusive diet of polished rice. The results were consistent and in the case of each of the two materials investigated, the curative dose had to be doubled after 1 hour's heating at temperatures around 120°C. (118 to 124°C.) and quadrupled after 2 hours exposure. The results of these experiments were supported by others obtained with commercial samples of tinned meat (Chick and Hume, 1916–17, p. 146), compared with raw meat. The minimum curative dose of the latter was found to be the equivalent

of about 30 gm. (dry weight), while doses of the former equivalent to 106 and 112 gm. (dry weight) respectively, in two samples were found to be insufficient for complete cures. Acting on these results the advice published was to the effect that "in case of armies and other populations subsisting largely on tinned foods, it is imperative to provide adequate supply of vitamine from outside sources." That this conclusion was justified is shown by the repeated occurrence of beri-beri among the British troops in the Dardanelles and Mesopotamia during the early part of the recent war (Willcox, 1916), when many individuals were forced to subsist for considerable periods on tinned meat, jam, and white bread (or biscuit baked from white flour). Had one-half the total diet consisted of tinned beans, it is improbable that beri-beri would have occurred, but a diet so constituted is not a reasonable proposition.

The criticism of our conclusions made by Daniels and McClurg upon the basis of work in which quantitative measurement was neglected cannot therefore be accepted. Any serious attack upon the problems concerned with the rôle of accessory food factors in which no attention is paid to quantitative measurement of daily intake and of required minima will inevitably lead to confusion sooner or later. For instance, had McCollum and Pitz (1917) in their investigation of guinea pig scurvy controlled quantitatively the amounts of milk and oats consumed daily by the individual animals in their experiments, they would probably have found a reasonable explanation of their results in the great variation existing between these amounts. It is possible therefore that these authors would not have been led to take up a position which was in opposition to most other workers in this field; *viz.*, that guinea pig scurvy was not a "deficiency disease" but was due to constipation following upon ingestion of a mechanically unsuitable diet. In this case, however, the confusion has been only temporary. The results of further experimental work have led McCollum[1] to abandon this conclusion and to adopt the more orthodox point of view that guinea pig scurvy, like human scurvy, is occasioned by lack of a specific vitamine in the diet.

Readers of the earlier researches on accessory food factors will appreciate the great value of nutritional work, which at that time

[1] McCollum, E. V., personal communication, 1919.

was of necessity only qualitative, but will also realize that many of the directly conflicting results obtained by different investigators were due to the use of different, but unmeasured, rations of the same foodstuff. Such confusion must necessarily arise in the study of a new subject, before the stage has been reached at which quantitative methods can profitably be applied. That stage has now been reached in the study of accessory food factors and it is imperative that the methods of work should include quantitative measurement in the future, if knowledge of this subject is to progress along trustworthy lines.

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MAINTENANCE AND REPRODUCTION WITH GRAINS AND GRAIN PRODUCTS AS THE SOLE DIETARY.*

By E. B. HART and H. STEENBOCK.

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(Received for publication, July 8, 1919.)

There is now available ample proof that mammals and fowls[1] cannot make normal growth if seeds or seed by-products constitute the sole articles of diet. To secure normal growth such food materials must be supplemented with minerals, with better proteins, and with the fat-soluble vitamine as in the case of the oat grain, barley grain, and especially the white corn grain as contrasted with the yellow corn grain.[2] For some mammals, as the guinea pig,[3] another supplement, the antiscorbutic vitamine, must be added, and probably for all species an addition of roughage is also necessary.

Though normal growth is impossible the question may now well be raised: Can normal *maintenance* and *reproduction* be secured when grains and their by-products constitute the entire dietary regimen? One might expect that for growth the demands for new construction would be too great to be met adequately by grains and grain products alone; and that when reproduction and especially rearing of the young are involved then a strictly grain ration would be inadequate,—although its non-efficiency might be obscured for a considerable length of time through the utilization of the reserve sources of the animal,—but that at least for main-

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[1] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373. Hart, E. B., Halpin, J. G., and Steenbock, H., *ibid.*, 1917, xxxi, 415. McCollum, E. V., and Davis, M., *ibid.*, 1915, xxiii, 181. McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916–17, xxviii, 153; 1917, xxix, 341.

[2] Steenbock, H., Boutwell, P. W., and Kent, H. E., *Proc. Soc. Biol. Chem.*, Apr. 24, 1919.

[3] Holst, A., and Fröhlich, T., *J. Hyg.*, 1907, vii, 634.

tenance the seeds would carry an ample supply of all chemical units needed for the repair of losses occasioned by the daily waste through metabolic activities.

For growth the mineral supplements to grains must be sodium, chlorine, and calcium.[4] In feeding practice as well as in human nutrition, the place of common salt in the diet is well established, but it is not a common practice to add salts of calcium. Where successful growth in practice is accomplished the abundant sources of calcium are not in the grains used, but in the grain supplements, such as milk, tankage (in animal feeding), plant parts other than seeds,—especially the stems and leaves—and natural waters.

To determine whether grains alone could maintain mammals, mature male rats were fed exclusively on ground wheat, ground barley, or ground corn (maize) and distilled water. Their curves of maintenance are shown in Chart 1. It is clearly evident that there cannot be normal maintenance of the mammal, even after maturity is reached, by the use of the seed alone. These animals became rough and scrawny in appearance, but their decline was slow, one of the group on corn as the sole article of diet living for more than 40 weeks. In these instances there must have been a tenacious retention of all the essential inorganic elements with minimum losses through the excretory channels.[5]

While normal maintenance with seeds is impossible, yet such dietary procedure is seldom, if ever, met with in practice. With certain classes of animals, as swine, it is not an uncommon practice to attempt their maintenance on grains alone, especially in the case of the winter breeding stock, but the grains and grain products used are usually supplemented with common salt and the salts common to natural waters. It was under such conditions of use that we desired definite information, because of the fact that such a program is not unusual among swine men and because of the possibility of a close correlation between the production of the still-born pig—through which the yearly losses to swine husbandry are large—and a dietary regime of faulty construction for breeding stock.

[4] Hart, E. B., Steenbock, H., and Fuller, J. G., *Research Bull. 30, Wisconsin Exp. Sta.*, 1914. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxx, 13. McCollum, E. V., *The Harvey Lectures*, 1916–17, xii, 151.

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 131.

EXPERIMENTAL.

For these experiments sows were used that had reached maturity on rations containing 15 to 25 per cent of alfalfa. The remainder of the ration came from the cereal grains, to which, in some cases, either peas, oil meal, or wheat middlings had been added. The specific ration used in the rearing of each lot of these animals is shown in the chart legends. After reaching weights of 200 to 300 pounds and the stage of maturity ready for breeding they were transferred to grains and grain products only. These animals were confined to pens with outside paddocks, the floors of which were of cement. The litter was shavings which swine do not eat. Common salt was added to the ration at the rate of 1 per cent. The only other salt addition to the ration was that furnished by the natural water used. This was a well water of some hardness, 1 liter containing 0.12 gm. of CaO. While daily water consumption varied, due to temperature fluctuations, we estimate that there was a daily consumption per individual of about 20 pounds which would be equivalent to an intake of 1.1 gm. of CaO.

Under these conditions of nutrition there was surprisingly good maintenance for considerable periods of time, although ultimately there was failure. Failure exhibited itself in the delay of estrum, failure to breed, production of an increasingly large percentage of dead pigs at birth, and in some cases there was total collapse of the animal, necessitating an immediate change in the ration. Recovery in most cases was immediate with the introduction of 25 per cent of ground alfalfa into the ration through which there was effected an improvement in the mineral, vitamine, and roughage content of the diet.

In some instances there was apparently normal maintenance with grains and grain product mixtures, common salt, and natural water for a period of 12 to 16 months, a not inconsiderable fraction of the normal span of life of this species. The average longevity of swine is estimated at 12 to 15 years. Probably the maintenance of live weight, which served as our guide in estimating whether or not we were dealing with *normal* maintenance, was not entirely a satisfactory criterion of maintenance since evidences of nutritional failure were exhibiting themselves in unsuccessful breeding and with increasing numbers of dead pigs at birth as the period of grain feeding progressed.

On corn 79 parts and peas 20 parts plus common salt and natural water (Chart 2) there was apparently normal maintenance for 15 months and at the present writing the animals appear to be in good condition. They were reared on a mixture of 49 parts of corn, 20 of peas, 30 of alfalfa, 1 of common salt, and natural water. Animal 1 had produced but two litters of young in this period, the first litter of nine being normal and strong, but the second litter numbered only four, three of which were born dead. Animal 2 also produced two litters of young in the 15 months, the first a litter of seven strong pigs and the second a litter of ten strong normal pigs. After the last litter was born there was failure to breed again for 5 months, but at the end of this time the animal was successfully bred and 4 months later produced a litter of three young weighing 7 pounds, two of which were alive and one dead. In neither case was the sow allowed to rear her young. It is altogether probable that first litter successes on grains alone were in part due to ample reserve stores accumulated by the mother during the pregestation period of normal nutrition.

With a ration of 49 parts of corn, 50 of whole oats, 1 of common salt, and natural water (the grains were always fed ground and mixed) the results were substantially in accord with those shown in Chart 2. Pronounced failure came only after 15 months of constant restriction to this ration (Chart 3); both animals produced strong and normal first litters, but the second litters contained large percentages of dead pigs. In the case of these sows the young were reduced to five in number and the mothers allowed to suckle them. The performance of these mothers was remarkable. The young grew at normal rates for 3 to 4 weeks (Chart 4), while the mothers lost rapidly in weight, decreasing 75 pounds in 3 weeks. Because of the tremendous losses in weight suffered by these sows, involving a large sacrifice of tissue for milk production, the suckling period was not extended over 5 weeks. It was apparent that what milk was secreted was normal in composition as evidenced by the initial normal rates of growth of the young; but it was also apparent that a ration of corn and oats could not sustain the live weight of a sow suckling five pigs growing at normal rates. It was evident that milk secretion and probably milk composition were maintained over this limited period of time through the supplementing powers of the mother's tissues and not

by the grains alone. Because of the great losses in tissue suffered by these sows and the probable decrease in milk flow it was impossible to suckle these young to weaning time. This was evidenced by the declining rates of growth of the young after a suckling period of 4 weeks. After the young were removed from the mother and mammary secretion stopped there was a gradual regain in weight by the sow to the preparturition weight.

On a ration of corn 69 parts, gluten feed 30 parts, common salt 1 part, and natural water there was maintenance of live weight and reproduction by both sows of fairly good first litters, but after a year on this ration evidence of inadequate nutrition appeared in the condition of the mothers (Chart 5). The second litter of young of No. 5 contained eight dead pigs at birth; in the case of No. 6 the pigs of the second litter were undersized and some were weak, while those of the third litter were immature and born 29 days ahead of time. Usually in these cases the young, although born dead and haired, are carried for the full gestation period. The sows used in this lot were reared on a mixture containing 74 parts of corn, 25 parts of alfalfa, 1 part of common salt, and natural water. No. 6 maintained her live weight for 19 months. This is the longest maintenance record on seeds or seed products that we have without evidence that collapse was imminent, necessitating a change in the ration.

The second litter of young from No. 6 was reduced to five pigs and this number of young was nourished with fair success for 4 weeks (Chart 6). Because of the great loss in weight sustained by the mother during this milk production period the ration was changed at the end of 4 weeks. There was another reason for changing the ration. While the mother, through the sacrifice of tissue, produced, in the early period of lactation, milk of normal composition as evidenced by the normal rates of growth of the young, later the growth of the young was not maintained at a normal rate. This may have been due to the scanty flow, or to an abnormal composition of the milk. We had no means of knowing what the true cause was, but it was evident not only from this case, but from other examples in our experimental group, that a lactating mother restricted to whole grains, grain mixtures, or grain mixtures reinforced with proteins of poor supplementing power such as used in this experiment could not carry large litters

of young at a normal rate of growth to weaning time; the weaning period for this species is 6 to 8 weeks.

With a more varied mixture of seeds and seed products no better maintenance than exhibited by the record of No. 6, Lot 3, could be obtained. Lot 4 was reared on a mixture of 69 parts of corn, 5 parts of casein, 25 parts of alfalfa, 1 part of common salt, and natural water. After reaching 300 and 350 pounds in weight, respectively, they were changed to a mixture of 29 parts of corn, 30 parts of oats, 30 parts of middlings, 10 parts of oil meal, 1 of common salt, and natural water. Chart 7 shows their records. No. 7 produced a good first litter, but a poor second litter and did not breed for some time thereafter, becoming stiff and unable to walk at the end of the restriction to this diet for 16 months. When changed to 74 parts of corn, 25 parts of alfalfa, 1 part of common salt, and natural water, stiffness disappeared and apparently perfect control of the limbs was regained. On this new ration this animal was successfully bred and reproduced a normal litter of seven young. No. 8 of this same lot (Lot 4) produced a first strong litter, a second litter of four, two of which were dead and two alive, and a third litter of seven pigs, all dead at birth.

Chart 8 shows an interesting record of the effect of a restriction to the corn grain alone, with natural water and common salt. The collapse of one of this group, No. 10, was much earlier than that of the other, earlier for both animals than where this grain was fortified with gluten feed, the protein-rich by-product of the corn grain. The longer maintenance on gluten feed plus corn grain is not to be attributed to a high calcium content of the gluten feed used. The CaO content of the gluten feed was but 0.027 per cent which is not higher than that of the whole maize kernel itself. The early collapse could not logically be attributed to a higher and superior protein mixture as there was a long maintenance of weight on the corn grain-oat grain mixture (Chart 3) which is neither appreciably higher in protein content than a straight corn ration, nor does it contain a protein mixture of superior worth for growth. This result is probably to be assigned to individual idiosyncrasies. The maintenance of live weight of No. 10 was secured for 5 months, at the end of which time she gave birth to a litter of young, some of which were below normal size and vitality. An attempt was made to have her suckle five of these young which

she did successfully for 2 weeks, but then became partially paralyzed, with cessation of milk flow. It was necessary to change the ration to 74 parts of corn, 25 parts of alfalfa, 1 part of common salt, and natural water on which recovery ensued. No. 9 maintained her live weight for 6 months, produced a first litter on this ration of eight young, five of which were dead and three alive. These three were weak and died a few days after birth.

To determine how successfully maintenance could be secured on a ration of seed and seed by-products plus natural water,—but to which no common salt was added—two animals of approximately 200 to 240 pounds weight were started on a mixture of 30 parts of corn, 30 of oats, 30 of middlings, and 10 of oil meal. These animals had been reared on a mixture of 74 parts of corn, 25 of clover, 1 of common salt, and natural water. They not only continued to grow, reaching weights of 375 and 400 pounds, but maintained an apparently normal condition for 12 months, gave birth to strong normal first litters, one of which, No. 11, contained but two pigs, while the other, No. 12, contained eleven pigs (Chart 9). Sow 11 produced a second litter of nine fairly good pigs, two of which died and five of which were nourished successfully for 4 weeks. In the case of both mothers the young (in the case of No. 12 reduced to five young) were successfully nourished for 4 weeks, indicating that the milk secreted during that period was normal in composition. Animal 11 lost heavily in weight during the suckling period. Undoubtedly these animals reinforced their scanty supplies of common salt provided in their diet by drinking some of their urine, thereby greatly delaying an earlier collapse.

We have been less successful in reproduction with swine restricted to the barley grain alone, fortified with natural water and common salt. Animals started at 275 pounds grew to 350 to 375 pounds with this grain plus common salt and natural water as the sole article of diet, but at the end of 4 months they showed signs of collapse (Chart 10). They did not crawl on their knees as is observed with swine restricted to grains during their most active growth periods, but walked with an unsteady, staggering gait, sinking down completely with prostration if at all hurried. During this period of imperfect nutrition both sows gave birth to young (the first litters) and in both cases the young were born dead. They were haired and of normal size. The condition of

the mothers was precarious and necessitated a change in the ration to 74 parts of barley, 25 parts of alfalfa, 1 part of common salt, and natural water. On this new ration there was rapid recovery to an apparently normal condition.

With a ration of 96.5 parts of barley, 2.5 of wheat gluten, 1 of common salt, and natural water somewhat longer endurance to such restriction was shown. No. 15, Chart 11, became paralyzed comparatively early, lasting but 6 months, although she gained somewhat in weight. She gave birth to one litter of young, all of which were haired, but dead. Their period of intrauterine life was normal. This animal died 1 month after parturition. The cause of death was unknown. Animal 16, after 5 months on this ration and in apparently normal condition, gave birth to six dead pigs. They were haired, some were normal in size, but others were mummified. For 10 months after giving birth to these young this animal maintained her weight, but failed to breed and passed into the "rolling gait" stage; appetite was lost, decrease in live weight began with ultimate prostration. At this point the ration was changed to 74 parts of barley, 25 parts of alfalfa, common salt, and natural water with rapid recovery to apparently normal condition. That the disturbances in all these cases were of nutritional origin and could be corrected rapidly by the use of a natural material is of very great interest and importance. With the rat[6] it has been shown that barley, when properly supplemented, may constitute 80 per cent of the ration and provide perfect nutrition. It is clearly evident that for long vigorous maintenance and reproduction the seed and seed products are deficient, even if common salt and natural water of relatively high calcium content are supplements.

Animal 17 likewise restricted to 96.5 barley, 2.5 wheat gluten, 1 of common salt, and natural water gained in weight from 325 to 435 pounds in a period of 6½ months, at which time she gave birth to her first litter of five *dead* pigs. All of these young were haired, and of normal size. This animal, shortly after the birth of the young, developed the "rolling gait" condition and died suddenly.

[6] Steenbock, H., Kent, H. E., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxv, 61.

Where mature swine were confined to whole oats, common salt, and natural water (Chart 12) the same experience as with the other grains was accumulated. Maintenance of live weight was possible, but reproduction of normal young was a failure. We maintained these two sows, Nos. 18 and 19, for 20 months and they appeared in fair condition throughout the experiment. No. 18, after 4 months on the ration, gave birth to a small litter (four) of normal young. She did not breed again for 10 months, but finally became pregnant and produced a litter of eleven haired, but dead pigs. Both sows in this lot had been reared on 74 parts of whole oats, 25 of alfalfa, 1 of common salt, and natural water. Sow 19 was able to maintain her live weight on the whole oat diet and recover losses in weight after the birth of a litter of young, but in her case as in the case of other individuals a considerable number of the young were born dead. In the first litter of nine pigs, six were dead; in the second litter of pigs, weighing 37 pounds, eight were dead; while in the last litter of nine pigs all were dead at birth (Chart 12).

Where oil meal (15 parts) was added to whole oats (84 parts) (Chart 13), common salt (1 part), and natural water, there was little, if any, improvement in the character of the ration for breeding purposes. Maintenance was possible as in the case of the use of whole oats alone, but a considerable number of the young were born dead. Animal 20 gained in weight on this ration, but produced a first litter of eleven haired, dead pigs. 5 months later she produced another litter of eleven dead pigs, weighing 22 pounds and partly hairless. Animal 21, put on the same ration at a weight of 280 pounds, gained rapidly in weight, reaching 390 pounds in 6½ months. At this time she gave birth to twelve pigs, five of which were born dead and seven alive. The living pigs were weak, all of them gradually growing weaker and weaker and in the course of 10 days all had died. Even after a lapse of 8 months it has not been possible to breed this animal again successfully, although she maintained her live weight.

DISCUSSION.

While these data indicate that under the practical conditions of their use maintenance can be secured with grains and grain products for a considerable length of time, the data likewise indicate

that by the sole use of grains and grain products complete nutrition is impossible.

The "practical condition of their use" implies supplementing the grains with common salt and natural water. Certainly the latter is a variable factor. In regions where the drinking water is soft the success attendant upon it as a source of supplementary mineral material to grains would be less than observed in these experiments. The outstanding feature of these experiments is the increasing mortality of the young with the progress of time under a constant restriction to such rations. The first litter may be a fair success, but quite invariably the succeeding litters will contain increasing numbers of *dead* pigs. The cause of this malnutrition is primarily an inadequate mineral intake. Roughage and vitamines may be of some importance in these experiments, but in rank of importance their relation is subsidiary to that of the mineral matter. We base this statement on the fact that we have had very good success in growing swine[7] on a ration made of corn-meal, gluten feed, and a proper salt mixture. With some of the grains, as oats and barley, the fat-soluble vitamine deficiency may be of greater importance than in the case of the yellow corn-meal used in these experiments.

The production of dead pigs at birth through inadequate nutrition is not different from our results with herbivora[8] where with a grain and a poor roughage, such as a cereal straw, immature and dead or weak calves are produced. This effect on cows is primarily a mineral deficiency. As the trouble can be corrected by the use of calcium salts imposed on a cereal grain-cereal straw-common salt diet[9] we likewise believe that the principal deficiency in the ration of these breeding swine is calcium and that the cause of dead pigs and dead calves from such rations as we have used is reducible to the same explanation.

Our working hypothesis does not assume that these calcium salts are needed primarily for constructive processes, such as bone formation and milk production, although we recognize the

[7] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373.

[8] Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Research Bull. 17, Wisconsin Exp. Sta.*, 1911; *J. Agr. Research*, 1917, x, 175; *Bull. 187, Wisconsin Exp. Sta.*, 1918.

[9] Hart, E. B., unpublished data.

extravagant intestinal waste of calcium by animals, but is an adoption of the view of Clowes[10] in regard to the function of calcium and sodium salts in altering the permeability of protoplasmic structure and in this specific instance the protoplasmic structure of the intestinal wall. This hypothesis would assume that with a preponderance of sodium salts in the diet and a low amount of calcium salts the surface protoplasmic films of the epithelial cells of the intestinal mucosa would present a structure in which water was the continuous phase. The permeability of such a system to water and water-soluble substances would be much greater than in a system where fat or lipoid material is the continuous phase. With the increase of calcium salts in the diet the proportion of calcium to sodium would change, establishing a condition favorable to the formation of a protoplasmic structure in which the fat or lipoid material would be the continuous phase. This latter system would be one of less permeability to water and water-soluble substances. This hypothesis of intestinal protoplasmic structure as influenced by the balance of sodium-calcium salts in the diet would pave the way for the view that on low calcium diets there would be especially favorable conditions for the continued absorption of products of intestinal origin, among which may be bacterial toxins or amines. Further, that an increased absorption of such products as mentioned would ultimately lead to physiological disturbances such as an undue stimulating effect on plain muscles as exhibited by certain amines; an undue stimulation to the central nervous system, causing tremors of the limbs and vasoconstriction; and an interference with the normal oxidative processes of the tissues leading to intoxication and asphyxiation. It is entirely conceivable that such conditions in the mother would lead to the production of weak or dead offspring. Some phases of the thesis outlined in this brief discussion are subject to experimental inquiry and will be dealt with as time permits.

SUMMARY.

These experiments deal with the maintenance of mature animals (rats and swine), and reproduction (swine). Growth, primarily, is not involved.

[10] Clowes, G. H. A., *J. Phys. Chem.*, 1916, xx, 407. Bancroft, W. D., *ibid.*, 1913, xvii, 501.

1. Whole grains fed as the sole article of the dietary and not supplemented with common salt or the salts of natural water will fail to support mature male rats.

2. Similar rations, including grain and grain by-products and fortified with common salt and a natural water of considerable hardness will maintain mature swine in an apparently fair condition of nutrition for many months, but ultimately nutritional failure will result.

3. No one grain was distinctly superior to another in respect to its capacity to maintain these animals, although nutritional failure exhibited itself earlier when barley was the sole grain used as compared with the corn or oat grain. This general and ultimate failure with all grains points to the inorganic content of the ration as the main deficiency.

4. Nutrition with seeds and seed products fortified only with natural water and common salt leads to increasing numbers of dead pigs at birth. In some cases the first litter produced was apparently normal, but imperfect nutrition was cumulative in its effect and increasing numbers of dead pigs at birth resulted in the second and third litters.

5. Dietary restriction to grains and grain products measurably reduced the breeding potency of this class of animals as seen in the complete disappearance of estrum or a delay in its frequency.

6. These results are compared with similar results we have secured with herbivora (cattle) restricted to a single grain and a roughage low in mineral content. The primary disturbing factor in both series of results is a low calcium intake, accompanied by a fair supply of sodium salts. A brief discussion of the hypothesis of permeability of the intestinal wall, as modified by the proportion of calcium to sodium salts in the diet is presented and this hypothesis used to explain our results.

Because of differences in the rates of growth and the rates of utilization of food constituents by different species we want to urge caution in the formulation of broad generalizations, applicable to all species, from the results secured with a single species.

CHARTS.

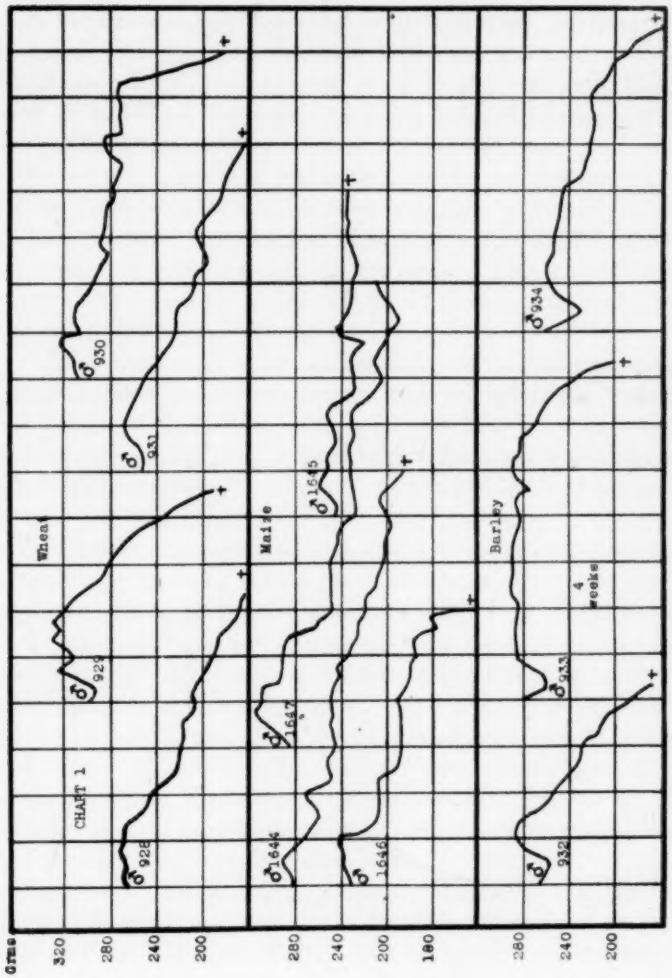


CHART 1. Illustrates the gradual decline of male rats strictly confined to a single grain and distilled water. The maintenance of those receiving the corn grain (maize, yellow corn) was somewhat better sustained than those on wheat or barley.

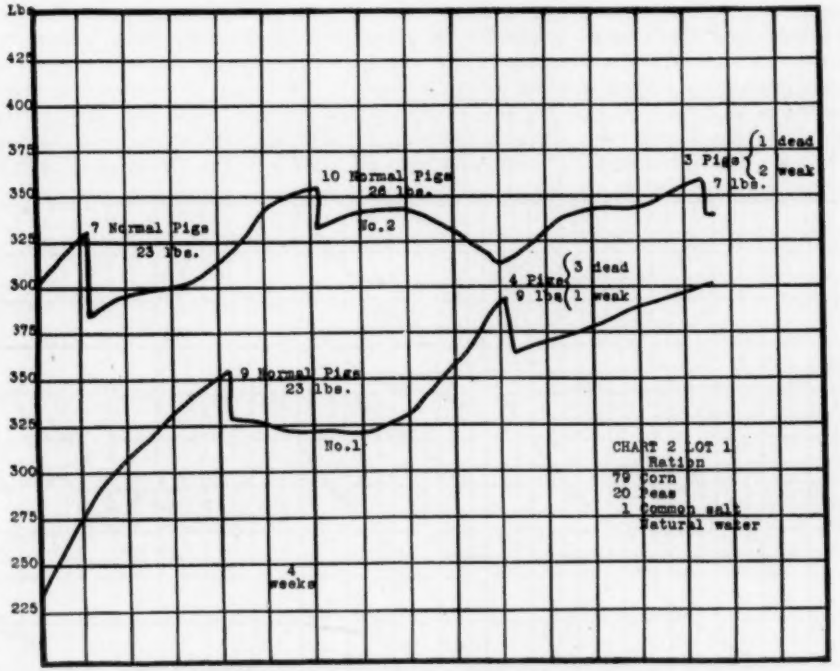


CHART 2. This chart shows the curves of maintenance of mature sows on 79 parts corn, 20 peas, 1 common salt, and natural water. They had been reared on a ration of 49 parts corn, 20 peas, 30 alfalfa, 1 common salt, and natural water. The use of common salt and natural water of a fair degree of hardness made possible a very long maintenance with these seeds, but ultimately there was failure of normal nutrition, which was especially exhibited in the increasing numbers of dead pigs at birth.

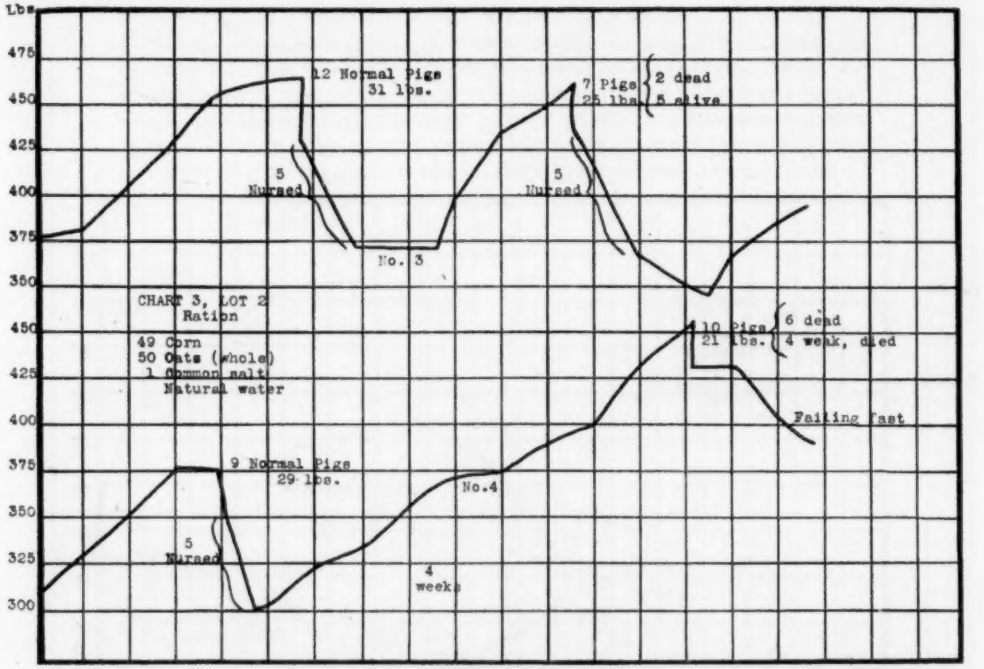


CHART 3. With a mixture of corn and oats, 1 per cent of common salt, and natural water maintenance of live weight was possible for many months. The first litters of young were normal in size and vigor, but the later litters were either smaller in number or contained a considerable number of dead pigs. Ultimately there was a decline in weight and extremely poor condition as seen in the curve of Animal 4. These animals had been reared on 19 parts corn, 20 oats, 20 middlings, 10 oil meal, 25 alfalfa, 5 starch, 1 common salt, and natural water. The young could be reared at a normal rate of growth over short periods of time (Chart 4), but not to weaning time.

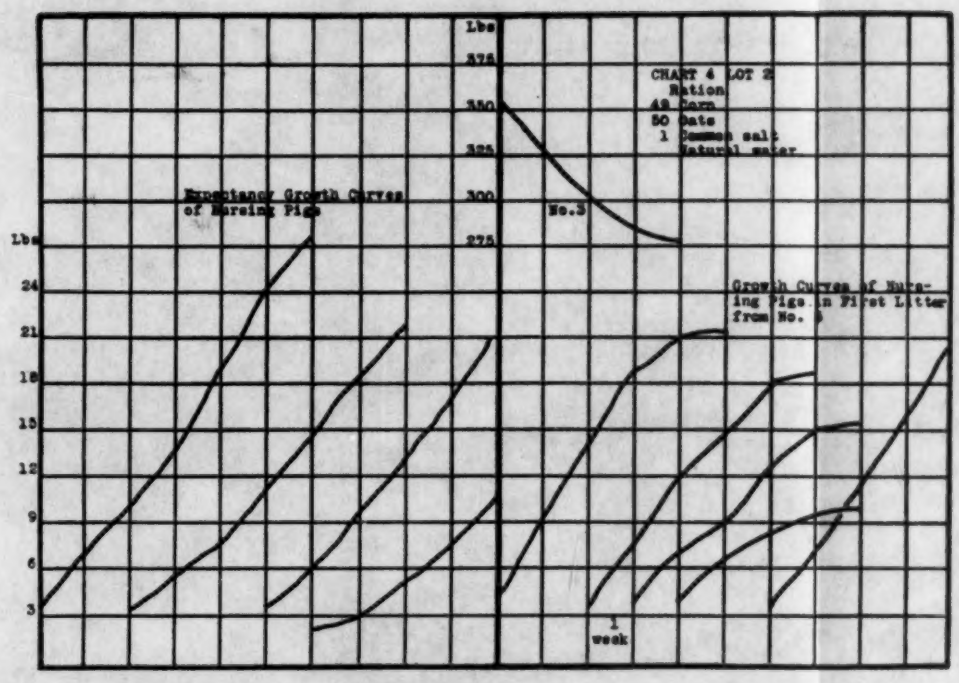
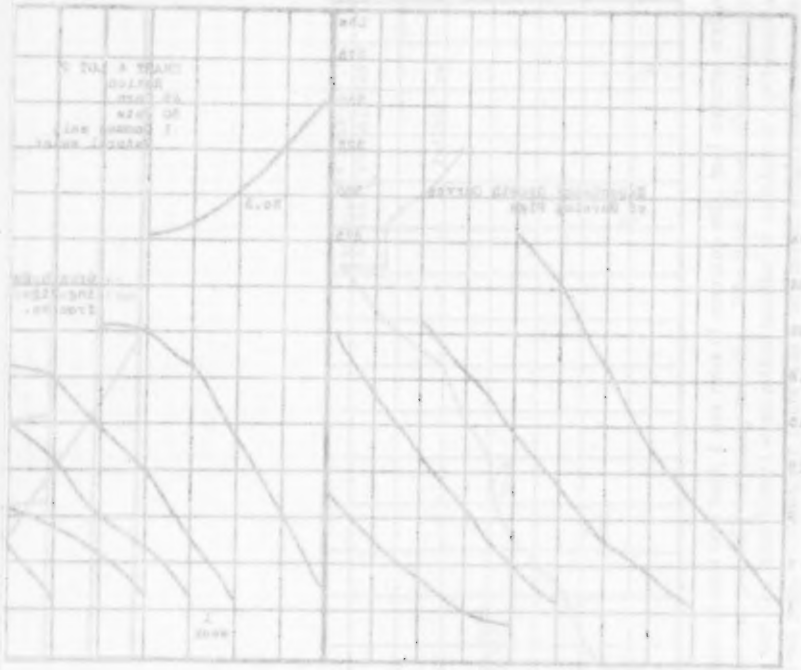


CHART 4. The left part of this chart illustrates the expectancy growth curves of normal suckling pigs. On the right of the chart are shown the curves of growth of five young suckled by Animal 3, Chart 3. On a diet of corn and oats this animal was able to suckle this number of young at a normal rate of growth for 3 to 4 weeks, but after that time their rate of growth declined. Accompanying the suckling period the mother lost weight at a rapid rate, which was regained after the young were removed. It is evident that what milk was secreted was normal in composition, although in the later period of suckling it may have been deficient both in quantity and quality. The latter supposition is doubtful as the young remained vigorous although their rate of growth had declined.



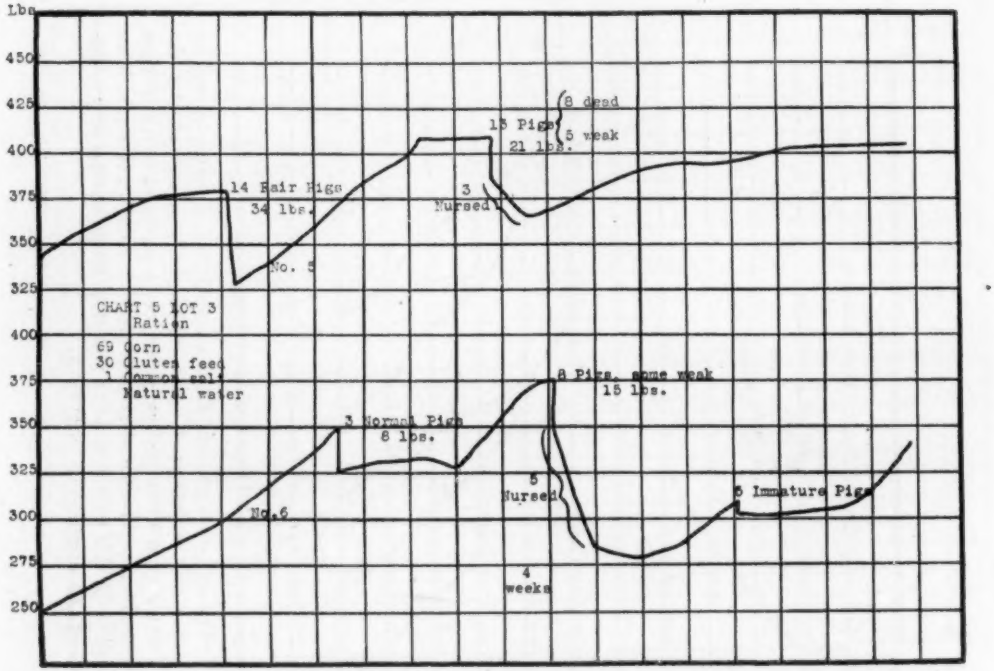


CHART 5. Where mature sows were restricted to a diet of 69 parts corn, 30 gluten feed, 1 common salt, and natural water maintenance for many months was possible, but reproduction was disturbed as shown by the increasing numbers of weak or dead pigs at birth. These animals had been reared on 74 parts corn, 25 alfalfa, 1 common salt, and natural water.

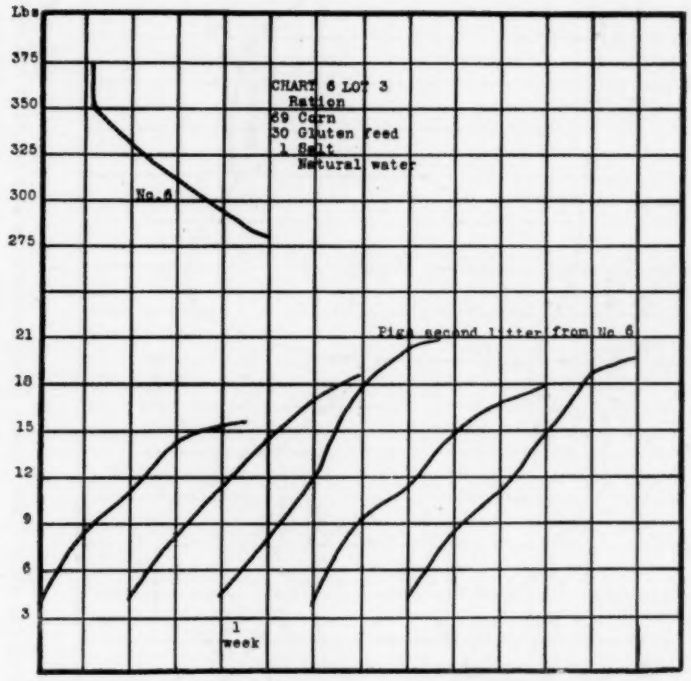


CHART 6. The curves of growth of the second litter of young nursed by Animal 6, Chart 5. While rapid losses in weight were suffered by the mother the young grew at normal rates for 3 to 4 weeks after which time there was a decline in the rate of growth on such a diet. It would have been impossible for this animal to have reared to weaning time an average litter of young at a normal rate of growth.

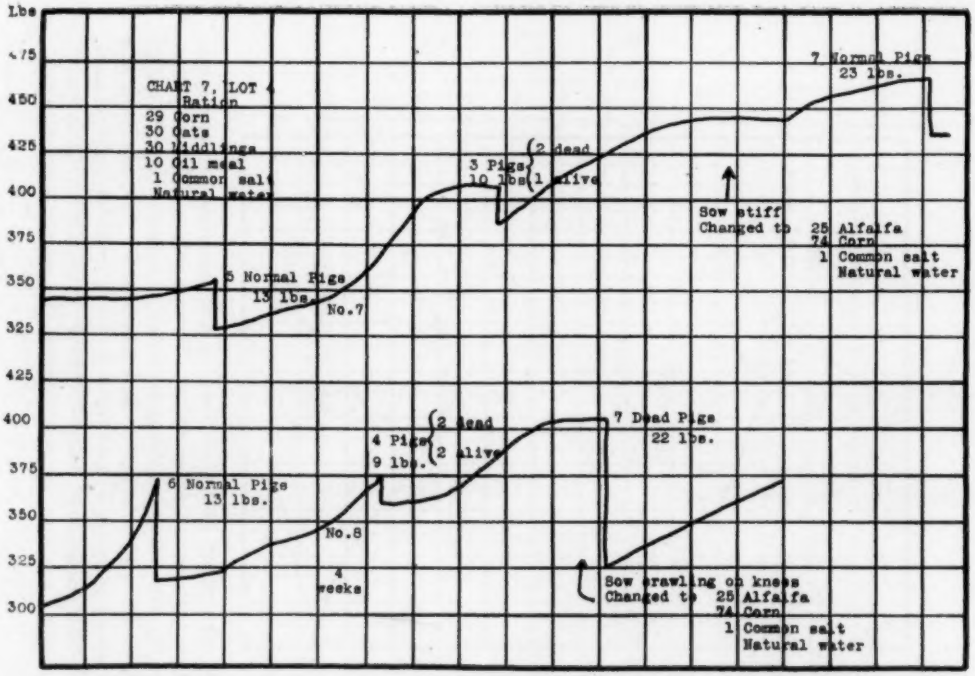


CHART 7. Where a more complex seed mixture was used there was no improvement in maintenance and reproduction. Live weight was maintained for 12 to 15 months. At that time collapse resulted and necessitated a change in the character of the ration. The first litter of young was normal, but later litters contained increasing numbers of dead pigs. These animals had been reared on 69 parts corn, 5 casein, 25 alfalfa, 1 common salt, and natural water.

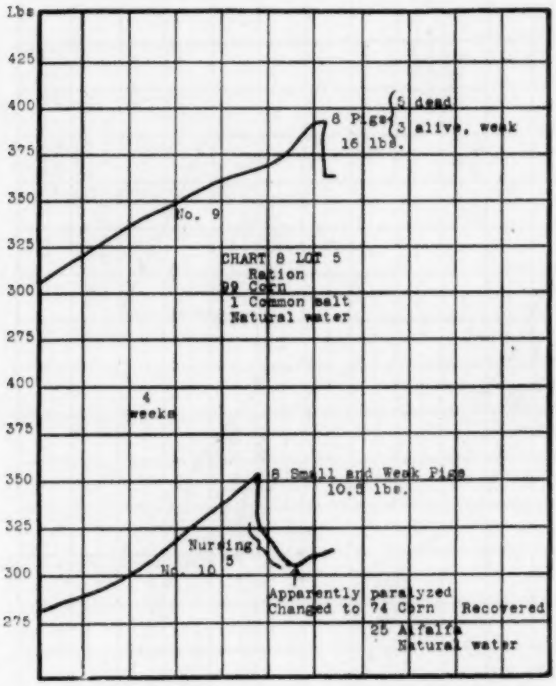


CHART 8. This chart illustrates the result of restriction to corn grain only, common salt, and natural water. There was maintenance of live weight, but the first litters of young were below normal in weight and 60 per cent of the litter from Animal 9 was dead at birth. These animals had been reared on a mixture of 24 parts corn, 25 oats, 25 middlings, 10 oil meal, 15 alfalfa, 1 common salt, and natural water.

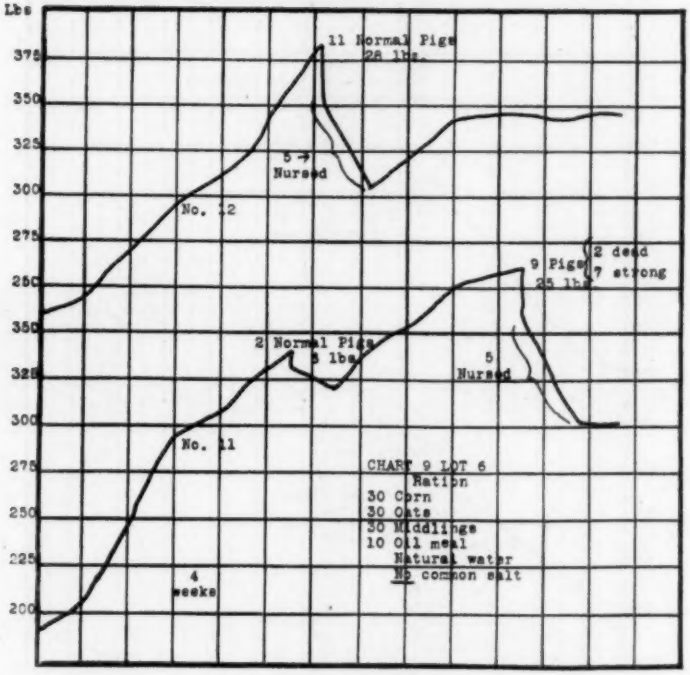


CHART 9. For these sows a mixed grain ration was used with natural water, but *no common salt was added*. There was remarkable maintenance and even considerable growth. The first litter of No. 12 was one of normal pigs. No. 11 produced two litters, the first small, but the second a litter of nine pigs, two of which were dead. These animals were inclined to continually drink some of their urine, thereby making good some of the losses in inorganic constituents. Both sows had been reared on 74 parts corn, 25 clover, 1 common salt, and natural water.

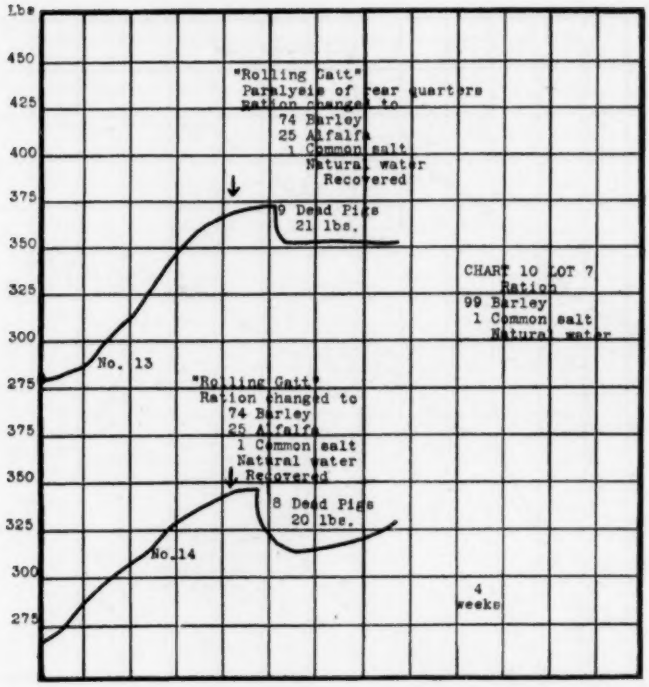


CHART 10. A ration of 99 parts barley, 1 common salt, and natural water was sufficient for maintenance for 4 months, but fatal to prolonged maintenance and successful reproduction. All the young in the first litters were born dead. Even before the young were born these animals developed an unsteady, staggering gait. It was necessary to change the ration shortly before the birth of the young. On the new diet containing 25 per cent of alfalfa recovery followed. These animals had been reared on a ration of 29 parts corn, 25 oats, 25 middlings, 10 oil meal, 10 alfalfa, 1 common salt, and natural water.

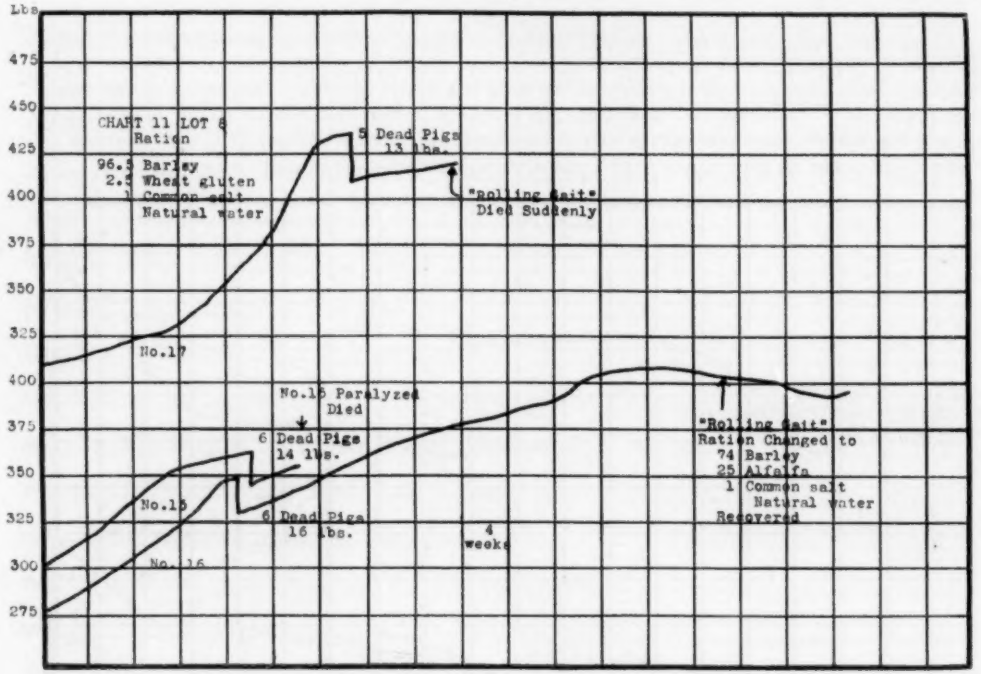


CHART 11. Another ration with a high barley content. On a mixture of 96.5 parts barley, 2.5 wheat gluten, 1 common salt, and natural water the results were practically a duplicate of those shown in Chart 10. The "rolling gait" stage developed comparatively early with Nos. 15 and 17. Both first litters were born dead. Animal 16 likewise produced a first litter of dead pigs, but maintained herself for several months longer in fairly good condition. Ultimately she developed the staggering gait condition, but recovered on a diet of 74 parts barley, 25 alfalfa, 1 common salt, and natural water. These animals had been reared on 74 parts wheat, 25 alfalfa, 1 common salt, and natural water.

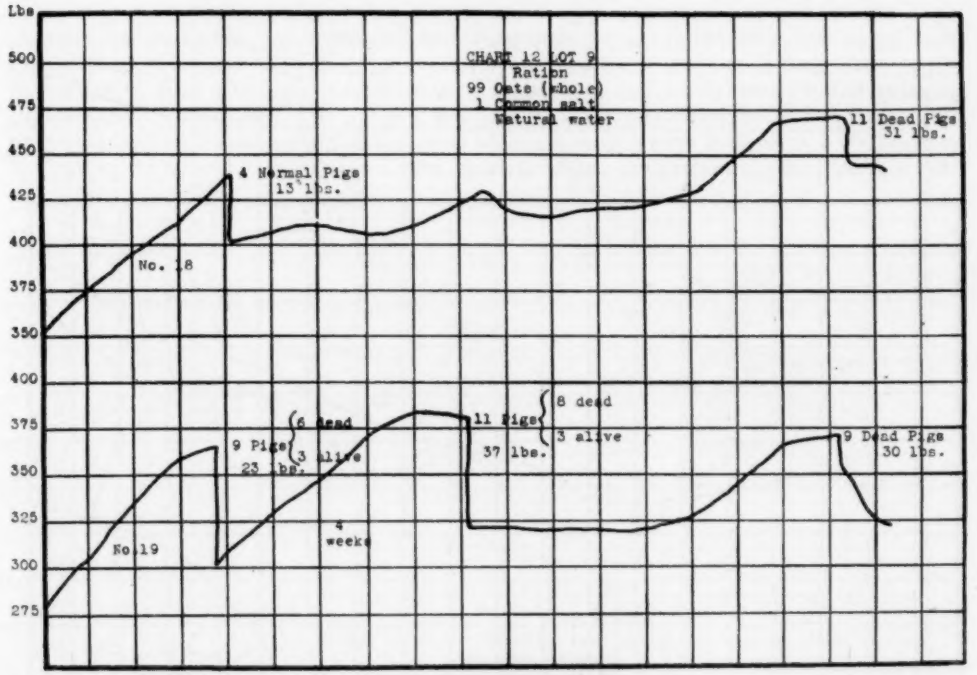


CHART 12. This chart illustrates maintenance and reproduction with 99 parts whole oats, 1 common salt, and natural water. Maintenance of live weight was surprisingly successful, but perfect nutrition was wanting as shown in the records of reproduction. The first litters were small while later litters were born at long intervals and contained increasing numbers of dead pigs. These animals had been reared on 74 parts whole oats, 25 alfalfa, 1 common salt, and natural water.

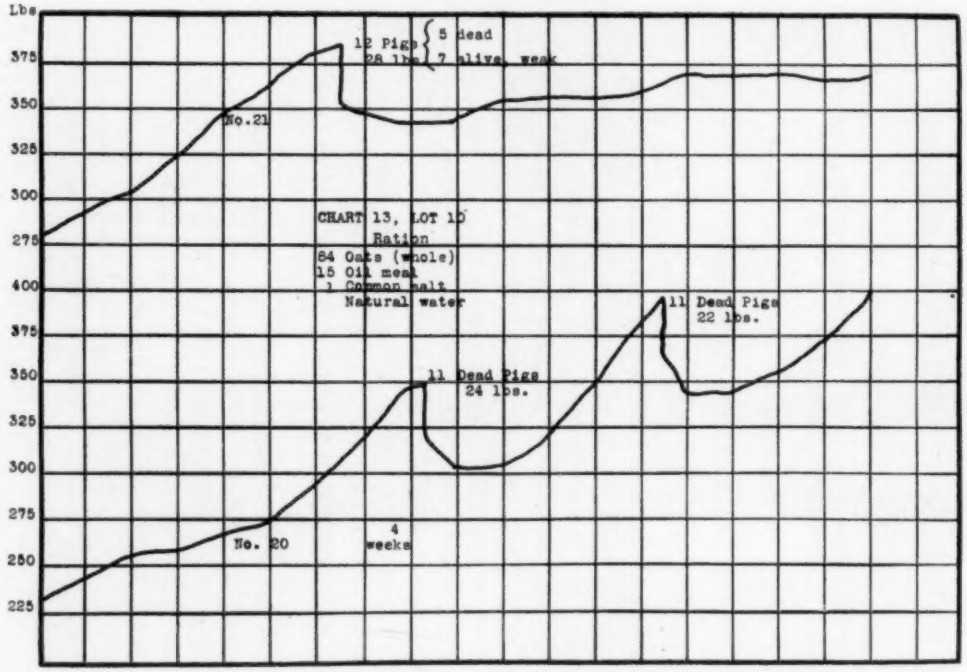


CHART 13. Fortifying the oat grain (84 parts) with oil meal (15 parts) did not improve the ration for continuous and adequate nutrition. Live weight was maintained over a very long time (18 months), but failure in reproduction was evident. The young were either weak or dead at birth. These sows had been reared on a mixture of 33 parts oats, 33 corn, 33 alfalfa, 1 common salt, and natural water.

VITAMINE REQUIREMENTS OF CERTAIN YEASTS.*

By FREDA M. BACHMANN.†

(From the Bacteriological Laboratory, Agricultural College, University of Wisconsin, Madison.)

PLATE 1.

(Received for publication, June 25, 1919.)

In the last few years it seems to have been satisfactorily proved by the work of Osborne, Mendel, McCollum, Funk, and others that a diet consisting of mixtures of purified proteins, fats, carbohydrates, and mineral salts is not sufficient for normal growth and reproduction in animals. In addition to mixtures of such purified foods, it is necessary to add certain substances which have been given different names such as vitamines, food accessory substances, growth determinants, and growth stimulants. There have been objections raised to the use of the name vitamine (11), nevertheless for lack of a briefer term and since the word has come to be used rather commonly, I shall refer to this class of substances in this paper by the use of this term.

Recent studies have shown that these chemically little known substances are necessary in the nutrition of other forms of life. Bottomley (2) found that the addition of raw peat, when this is further decomposed by means of aerobic soil organisms, to a mineral culture solution in which *Lemna minor* plants were growing, resulted in a great increase in the number and weight of the plants. The remarkable effect of the addition of very small amounts of such organic substances to the mineral culture solu-

* Published with the permission of the Director of the Wisconsin Agricultural Station.

† In collecting the necessary data for the results recorded in this paper I have had the help of several thesis students: Helen M. Reed, Genevieve D. Moore, Reba C. Haner, Katharine Hudson, Marguerite Beattie, and Mariana T. Sell.

tions may show a need of vitamines by the green plant. Bottomley suggests that "some of these growth-promoting substances may act directly as organic nutrients, and others may be of the nature of accessory food substances." It is a well known fact that certain of the pathogenic bacteria produce either a very scanty growth or no growth at all when transferred to many of the kinds of culture media used commonly in bacteriological laboratories. If certain of the body fluids are present these organisms produce abundant growth. Davis (5) has shown that in the addition of hemoglobin to culture media for the growth of hemophilic bacteria, we are adding a substance the action of which is strikingly similar to that of the vitamines required for animal nutrition. Lloyd (10) has shown that such substances are necessary for the successful cultivation of the meningococcus. Cole and Lloyd (4) have found in their studies of the nutrition of the gonococcus that vitamines are also necessary. Mockeridge (12) found that the presence of soluble humus, especially that produced by bacterial decomposition, in the soil increases the rate of nitrogen fixation and nitrification and that the effect of these soluble decomposition products upon the nitrogen-fixing and nitrifying bacteria is due to the presence in the humus of growth-promoting substances or vitamines.

According to some of the observations of Pasteur (13) it seemed that yeasts might differ from many organisms in this need for vitamines. He found that solutions of inorganic salts plus sugar would ferment with an increase in number of the fermenting organisms. In 1901, Wildier (14) published the results of some studies on fermentation. He maintained that some other element must be added to Pasteur's solution for the cultivation of yeast if the amount of the inoculum was small. If the amount of the inoculating material was large fermentation soon followed. This according to Wildier was supposedly because there was introduced with the larger inoculum some substance which was readily soluble in water, and absolutely essential for fermentation with yeast. Wildier terms this indispensable substance "bios." Amand (1) continued Wildier's studies and proved that the failure to secure fermentation with light inoculations of yeast was not due to any toxic substance in the medium.

A few years ago while experimenting with two strains of yeast isolated from a commercial yeast (Yeast Foam), I was able to confirm, in part at least, the observations of Wildier. My yeasts which I have numbered 3 and 5 were grown in streak cultures on potato agar. From these streaks large amounts of yeast could be obtained for inoculation of the inorganic medium. A platinum loopful of the yeast from an agar culture was used for a heavy inoculation. A lighter inoculation was obtained by introducing one loopful of yeast cells into 15 cc. of sterile water and then using one loopful of this suspension. Two kinds of inorganic media with dextrose added were used. The composition of these was as follows:

A. 100 cc. distilled water.
1 gm. ammonium tartrate.
1 " yeast ash.
10 " sugar (dextrose).

B. 838 cc. distilled water.
10 gm. ammonium tartrate.
0.2 " magnesium sulfate.
0.2 " calcium phosphate.
2 " potassium acid phosphate.
150 " dextrose.

The results obtained with these two kinds of media were similar.

Fermentation tubes containing approximately 20 cc. of medium were used as a means of determining whether gas was produced. The temperature of incubation was 28°C. With the lighter inoculation of yeast cells into either of the inorganic media no gas was produced until the cultures were at least 1 week old. With the heavier inoculation some gas was produced within 3 days. There seemed to be something introduced in the heavier inoculation which made the medium more favorable for the growth of the yeast cells and for fermentation. At the suggestion of Dr. Amy Daniels that the delay in fermentation with light inoculation might be due to a lack of sufficient vitamine content in the medium, a small amount of water-soluble B was added to the medium in the fermentation tubes. The solution of vitamine was prepared as follows: The white pulp from the peel of four oranges was boiled with two cups of water until evaporated to one-half the original volume. The liquid was then de-

canted off and sterilized in the autoclave. When 2 cc. of this solution were added to the medium in the fermentation tubes, fermentation took place considerably sooner. The tubes receiving a heavy inoculation of yeast plus 2 cc. of the orange peel extract showed gas formation in 36 hours as contrasted with 72 hours when no extract was added. The tubes receiving the lighter inoculation of yeast plus 2 cc. of the extract from the orange peel showed gas in 2 days as contrasted with 7 days when no extract was added (Plate 1). These experiments were repeated several times with the same results so that I was convinced that the inorganic culture media which I was using plus sugar were not entirely favorable for fermentation with these yeasts and that the addition of an extract from the white pulp of orange peel to such media greatly accelerates gas production. The results with the two strains of yeast were similar. It seemed probable that yeasts like many other organisms require a certain amount of vitamines. It may be that in a heavy inoculation some of the cells are dead, or that because the medium is unfavorable some may die and that from these dead cells soluble substances that are stimulating to growth and fermentation of other cells diffuse out into the medium.

For most of my later studies of the nutritive requirements of yeasts, I have used two other strains of yeast which I have designated by the numbers 49 and 51. Yeast 49 was isolated from the commercial Yeast Foam and was probably *Saccharomyces cerevisiæ*. Yeast 51 was isolated from canned pears which were fermenting. There is little difference in the microscopic appearance of these yeasts but their ability to ferment an inorganic culture medium containing dextrose is quite different. Both these yeasts grow well on potato agar and during the time I was experimenting with them I kept them in culture on this medium.

The liquid medium for testing the vitamine requirements of these yeasts was in every case Nägeli's solution. This has the following composition:

100	cc.	distilled water.
10	gm.	dextrose.
1	"	ammonium nitrate.
0.05	"	calcium phosphate.
0.5	"	potassium acid phosphate.
0.25	"	magnesium sulfate.

The distilled water was obtained by distilling the city water (from deep-driven wells) from an alkaline solution through an acid solution and again through alkaline solution. It has given conductivity tests of 2.77×10^{-6}. The dextrose used was obtained from the J. T. Baker Chemical Company, Philadelphia, Pa. None of the salts has been especially purified by recrystallizing. All apparatus used, *i.e.* test-tubes, vials, and fermentation tubes, has been thoroughly freed from any trace of organic matter by the use of a cleaning solution of potassium dichromate and sulfuric acid, and then thoroughly rinsed with distilled water. Durham tubes were usually used to show gas production and the amount of gas produced. Approximately 8 cc. of the medium were placed in these tubes. For some experiments Smith's fermentation tubes were used.

It was stated above that Yeasts 49 and 51 differ greatly in their ability to ferment Nägeli's solution. To show this, light and heavy inoculations of the yeast were introduced into the medium in Durham tubes. Six suspensions of yeast cells in sterile water were used for inoculation. Suspension 1 consisted of a loopful of yeast from an agar slant culture transferred to 10 cc. of sterile water. Suspension 2 consisted of 0.5 cc. of Suspension 1 introduced into 10 cc. of sterile water. Suspensions 3, 4, 5, and 6 were prepared similarly; *i.e.*, by each time adding 0.5 cc. of the less dilute suspension to sterile water.

Suspension 1 contained			1	loopful of yeast cells.			
"	2	"	$\frac{1}{20}$	"	"	"	"
"	3	"	$\frac{1}{400}$	"	"	"	"
"	4	"	$\frac{1}{8,000}$	"	"	"	"
"	5	"	$\frac{1}{160,000}$	"	"	"	"
"	6	"	$\frac{1}{3,200,000}$	"	"	"	"

The inoculations consisted of 0.5 cc. of each suspension. Triple inoculations of each suspension were made for comparison. With such inoculations results were obtained with Yeast 49 as recorded in Table I.

It should not be inferred from Table I that the lighter inoculations of yeast such as those in Suspensions 5 and 6 produce 10 per cent gas in the same time as the heavier inoculations. In this experiment, 2 weeks after inoculation there was 100 per cent gas in all the tubes but with the heavier inoculations 100 per cent gas was produced considerably before 2 weeks. It will be evident from this table that the amount of gas in the

TABLE I.

Suspension No.	Tube No.	Amount of gas after	
		8 days.	15 days.
		per cent	*per cent*
1	1	60	100
	2	50	100
	3	100	100
2	1	63	100
	2	60	100
	3	60	100
3	1	25	100
	2	30	100
	3	10	100
4	1	5	100
	2	5	100
	3	15	100
5	1	Bubble.	100
	2	"	100
	3	4	100
6	1	Bubble.	100
	2	"	100
	3	"	100

triplicate tubes supposedly with the same inoculation varies after a week's incubation. One explanation of this may be that exactly the same number of cells was not introduced into each tube. Yeast cells are so large that in a suspension in water they very soon fall to the bottom of the container and unless great care in shaking the suspension is exercised the suspension of cells is not uniform.

It is probable that No. 3 inoculated from Suspension 1 received a heavier inoculation than Tubes 1 and 2. It is probable, however, that there was little difference in the inoculation of Tubes 1 and 2. When fermentation with yeast is once started, gas may be formed so rapidly that the difference in time between the formation of 50 and 60 per cent of gas may be less than 1 hour.

The eighteen tubes inoculated from the six suspensions of Yeast 51 showed no gas at all after 2 weeks incubation. Usually such cultures with Yeast 51 were discarded after 2 weeks, but occasionally they were incubated for 4 or 6 weeks without any gas being produced. Yeast 51 then is physiologically different from No. 49 in that it does not ferment Nägeli's solution even in heavy inoculations.

Table I shows the same results as those recorded above for Yeasts 3 and 4. With these three strains of yeast the time be-

TABLE II.

Yeast No.	Number of cells introduced.	Time incubated.	Gas.
		days	*per cent*
49	52,800	9	95
51	300,000,000	14	0

fore which fermentation begins varies with the number of cells introduced. All three of these yeasts will ferment an inorganic medium although the fermentation is much more rapid if some material rich in vitamines is added. They are apparently either able to synthesize the necessary amount and kind of vitamines after sufficient time or they are capable of growing in a medium containing a small amount of vitamines.

The smallest number of cells of Yeasts 49 and 51 necessary to cause the production of gas when these are introduced into a given amount of Nägeli's solution, was not determined. However, the number of cells per cc. in some of the suspensions used for inoculation was determined by plating a measured amount of the suspension with potato agar in Petri dishes. In this way the approximate number of cells introduced in fermentation tubes of the medium was determined with results as recorded in Table II.

These two yeasts differ in their relation to oxygen. Yeast 49 always forms a heavy grayish white surface film with some clouding of the medium. Later this film sinks. Yeast 51 produces a finely granular sediment and leaves the medium clear. Dutcher (7) has shown that the catalase content of tissues is lowered in pigeons fed on a diet deficient in water-soluble B. He suggests that either the disease is accompanied by incomplete or partial oxidation, with the accumulation in the tissues of products of incomplete oxidation or that water-soluble vitamines function in some way to stimulate oxidative processes. In this connection it is interesting to observe that Yeast 49, which readily ferments Nägeli's solution without the addition of any vitamine, is apparently aerobic in its oxygen requirements and Yeast 51 which does not produce a surface film is unable to ferment the medium unless vitamines are added. It may be that if Yeast 49 does synthesize its own vitamines, this synthesis is in some way connected with oxidative processes. Water-soluble B is especially abundant in the leaves of plants and while the leaves are the organs of assimilation they are also important as the organs of respiration.

To a limited extent, multiplication of cells in Yeast 51 does not seem to be necessarily associated with gas production. The sediment in the neck of the fermentation tube is a mass of yeast cells. These, however, do not appear to be vigorous, healthy cells as seen with the microscope. They are not dead because when given favorable conditions such as a change in the medium as I shall describe later, fermentation follows. It is entirely possible that we have in these media of inorganic substances and dextrose, media which are not absolutely vitamine-free and that the amount which is present is sufficient for initiating fermentation with Yeast 49 and not with Yeast 51. As stated above there was no attempt to purify the salts by recrystallization or any attempt to purify the sugar. Whether the medium is vitamine-free or not, it is very evident that there is a great difference in the need of these two yeasts for traces of organic substances.

While Pasteur (13) obtained fermentation in inorganic solutions to which sugar was added he also found that there was a great difference in the amount of growth obtained with a beer yeast

depending on whether an abundance of organic matter was present or not. This statement of Pasteur's seems to have been overlooked by Wildier (14). In my studies of Yeasts 49 and 51, I have been able to confirm the observations of Pasteur that certain yeasts will grow in and ferment a medium composed of water with inorganic salts and sugar. I have also been able to confirm the observations of Wildier that certain yeasts (as my Yeast 51) when introduced in small numbers will not ferment a medium of inorganic salts, sugar, and water, and also I have been able to confirm Pasteur's statement that the addition of some organic matter accelerates fermentation.

Since Yeast 51 will not ferment Nägeli's solution some experiments were done to determine if perhaps the addition of traces

TABLE III.

Tubes containing yeast extract.		Tubes without yeast water.	
5 days.	21 days.	5 days.	21 days.
per cent	*per cent*	*per cent*	*per cent*
100	20	0	5
100	30	0	15
100	25	0	0
100	25	15	Bubble.
100	25	0	0
100	25	0	0
100	35	0	0
100	50	0	0

of organic matter would stimulate gas production. Wildier (14) found that fermentation took place readily if a small amount of yeast extract was added to the medium. Hence yeast water was tried first. This was prepared by boiling baker's yeast in water until it was of a thin syrupy consistency. It was then tubed, plugged, and sterilized in the autoclave at 15 pounds for 15 minutes. Sixteen tubes of this medium were heavily inoculated from a 72 hour streak culture of Yeast 51 on potato agar. To eight of these tubes one drop of yeast water was added. The amounts of gas in per cent produced after 5 and 21 days incubation are given in Table III.

From this table it may be seen that yeast water in the amount introduced furnished the necessary amount of organic matter or

vitamines to enable the yeast to ferment the medium. The amount of gas in the tubes to which yeast extract was added was very much less after 21 days than after 5 days. This is because the CO_2 produced is very readily absorbed by the medium. After 5 days incubation there was gas in only one tube of those to which no yeast extract had been added. The maximum amount of gas produced was 15 per cent. The fermentation in this tube did not continue. The small amount of CO_2 produced was almost completely absorbed after 2 weeks so that only a tiny bubble remained. Gas was produced after 21 days in two other tubes to which no yeast extract had been added, but again this was the maximum amount of gas. The cultures were kept 4

TABLE IV.

Inoculum.	Tube No.	Gas in medium.	
		Containing yeast extract.	Without yeast extract.
		per cent	*per cent*
1 loop of agar culture.	1	100	20
	2	100	20
1 drop of Suspension 3.	1	100	0
	2	100	0
	3	100	0
1 " " " 4.	1	90	0
	2	95	0
	3	90	0

weeks. After this time five of the tubes without yeast extract showed no change whatever and in the three in which fermentation had begun, the gas was partly or almost entirely absorbed.

The experiment was repeated adding 2 cc. of the yeast extract to 500 cc. of the medium before it was tubed and sterilized. Very heavy and lighter inoculations of yeast were made. The heavy inoculations of yeast were directly from agar slant cultures, the lighter from suspensions of yeast cells in sterile water prepared as described above. The results were similar after 5 days incubation of the culture and are given in Table IV.

The results when peptone was added to the medium to the amount of 1 gm. for every 100 cc. of the medium were entirely

similar to those obtained with yeast extract. The inoculations were very heavy, taken directly from an agar slant culture. The maximum amount of gas in the tubes to which no peptone was added was 45 to 65 per cent after 8 days incubation. Later this gas was absorbed. In the tubes containing the peptone there was 100 per cent of CO_2 after 8 days.

Beef extract was used to determine its value as a stimulant for growth and fermentation with Yeast 51. In this case the inoculations were very light as compared with those used in the experiments with yeast extract and peptone. Five suspensions were prepared in the manner described above. The amount of inoculating material was one drop (approximately 0.05 cc.) of each

TABLE V.

Gas after 5 days.

Tubes containing beef extract.	Tubes without beef extract.
per cent	*per cent*
100	0
100	0
100	0
100	0
100	0
100	0
100	0
100	0
100	0

of these suspensions from a 1 cc. pipette. Nine tubes contained 0.25 gm. of Liebig's beef extract in 100 cc. of the medium. Nine other tubes contained no beef extract. Triplicate inoculations were made giving results as shown in Table V.

The results of these experiments are again in agreement with the observations of Pasteur, that the addition of small amounts of organic substances to a medium composed of salts and sugar in solution greatly stimulated the development of certain yeasts. The amount of the organic material added to the medium is so small in proportion to the remarkable effect upon the rate of fermentation by the yeast and the need by the yeast for such material is so imperative that it appears we have here among

some of the *Saccharomycetes* a vitamine need not unlike that of animals. At this point it seemed desirable to test further the similarity or dissimilarity of some of the vitamines necessary for yeasts and animals.

The study of these substances so necessary to a perfect diet for animals has been furthered almost entirely through the use of laboratory animals, rats, rabbits, and pigeons. Such experimentation has required much time, labor, materials, and expense. If the same substances were found to supply the vitamine requirement for both yeasts and animals some much needed studies of these latter substances might be greatly simplified. To determine whether a substance contains a necessary vitamine it should be fed to two or three generations of rats or other animals. If certain yeasts could be used it would be possible to gain the same amount of information in a few weeks.

Vegetable juices (8) are known to be rich in water-soluble B, hence it seemed desirable to determine the value of these as vitamines for Yeast 51. 35 gm. each of cut cabbage, potato, beet, and parsnip were placed in 175 cc. of distilled water in flasks for 4 hours. These were then heated for 40 minutes in a steamer at 100°C., and the liquid was decanted off and sterilized at 15 pounds pressure for 15 minutes. 0.5 cc. of each extract was added to 10 cc. of Nägeli's solution in Durham tubes. Only one suspension of yeast cells was used for inoculating the tubes. A loopful of Yeast 51 from an agar slant was transferred to a 10 cc. water blank and one loopful of this suspension used for inoculation. Triplicate tubes were prepared. Frequent examination of the cultures showed that the maximum amount of gas was produced with potato and beet after 10 days, and with cabbage and parsnip after 26 days. The results are given in Table VI.

The control tubes in which there was no vegetable extract showed no fermentation. The amount of juice which is extracted from the vegetable must vary with the treatment to which the vegetables are subjected. To extract more of the juices it seemed advisable to leave the cut vegetables longer in the distilled water. The experiment was repeated as follows: 100 gm. of each vegetable were placed in 500 cc. of distilled water in a refrigerator. After 48 hours the receptacles containing the vegetables and water were placed in the steamer and kept at 100°C.

for 45 minutes. The vegetable extract was then decanted off and sterilized at 15 pounds pressure for 15 minutes. Extracts were prepared from potato, carrot, celery, beet, onion, rutabaga, cabbage, cauliflower, parsnip, and lettuce. 0.2 cc. of these extracts was added to 10 cc. of the sterile Nägeli's solution in the Durham tubes. Three suspensions of yeasts prepared as described above were used for inoculation. Triplicate cultures were made with each. Tubes of media to which no extract of the vegetables was added served as controls. The amount of gas in per cent produced in the tubes is given in Table VII. The con-

TABLE VI.

Tube No.	Extract.	Amount of gas after	
		10 days.	26 days.
		per cent	*per cent*
1	Potato.	80	50
2	"	50	20
3	"	70	40
1	Cabbage.	50	75
2	"	15	75
3	"	60	50
1	Beet.	0	0
2	"	0	0
3	"	20	15
1	Parsnip.	90	80
2	"	25	35
3	"	30	60

trol tubes with no extract showed no gas after 14 days. All the extracts furnished the necessary substance for the yeast to grow and ferment the medium even though the amount added was very little. The figures do not represent the maximum percentage of gas which was produced but merely the amount at the time this reading was taken. The maximum amount was produced after different periods of incubation with the different extracts.

As seen in Table VII the extracts were not equally potent. The potency of an extract should be determined by the time before fermentation begins in the medium and also by the maxi-

mum amount of gas which the yeast is able to produce. In the tubes containing onion and rutabaga extracts while there was considerable gas after a week's incubation no more gas was produced after another week. With carrot and celery extract the fermentation was somewhat more delayed but continued longer so that at the end of the 2nd week there was slightly more gas in these tubes than in those with the onion and rutabaga. The extracts of cabbage, cauliflower, parsnip, and lettuce seemed to have the greatest potency for these not only fermented rather quickly but in several of the tubes 100 per cent of gas was produced. In only one of the tubes with the cabbage extract was

TABLE VII.

Suspension No.	Tube No.	After 7 days.				After 8 days.					
		Potato.	Carrot.	Celery.	Lettuce.	Beet.	Onion.	Rutabaga.	Cabbage.	Cauliflower.	Parsnip.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	1	90	5	5	75	50	40	30	60	60	80
	2	40	10	10	60	55	45	35	60	80	70
	3	60	5	5	70	50	40	35	55	100	75
2	1	65	5	0	65	45	35	35	60	75	80
	2	50	5	0	60	40	30	20	75	50	90
	3	45	5	0	55	50	30	30	50	45	95
3	1	15	0	0	70	0	5	0	70	55	30
	2	20	0	0	60	0	10	0	75	50	25
	3	15	0	0	65	0	5	0	80	45	15

there 100 per cent of gas. Several of the tubes with the potato extract showed 85 and 90 per cent of gas after 10 days or 2 weeks.

The experiment was repeated to determine more exactly, if possible, the comparative value of the vitamines in the vegetable extracts. Since in the preceding experiment gas was produced in all the tubes to which vegetable extracts had been added, it was decided to add a smaller amount of the extracts. One drop (about 0.05 cc.) of each extract (except that of cauliflower) was added instead of 0.2 cc. as previously used. The medium was inoculated as before with Yeast 51. The results indicated that

there were not sufficient vitamines in one drop of any of the vegetables except the cabbage to stimulate the yeast to ferment the medium in 2 weeks. The cabbage extract therefore appeared to contain the most efficient vitamines for the yeast. In Table VIII is recorded the percentage of gas produced in the tubes containing cabbage extract. Cabbage is fed commonly to laboratory animals to prevent or cure polyneuritis. Lettuce and other vegetables are also used. In this ability of Yeast 51 to utilize extracts from these vegetables it appears to be very similar in its vitamine needs to the needs of animals.

TABLE VIII.

Suspension No.	Tube No.	Gas produced after	
		7 days.	15 days.
		per cent	*per cent*
1	1	5	50
	2	10	75
	3	5	70
2	1	10	70
	2	10	65
	3	5	60
3	1	5	20
	2	0	35
	3	5	40

Milk is also known to supply abundantly the vitamine needs of animals. To determine its value for Yeast 51, raw milk was obtained under as nearly aseptic conditions as possible. One drop of this was introduced into the tubes of Nägeli's solution inoculated with Yeast 51. Table IX shows that milk is likewise effective in stimulating growth and fermentation of this yeast.

The control tubes in this experiment were tubes to which no milk was added and also, to prove that the fermentation was not due to organisms in the milk, a set of tubes of Nägeli's solution to which one drop of milk but no yeast was added. In neither of these sets of controls was there any gas produced.

Dutcher (6) found that honey was deficient in water-soluble B. He fed pigeons on a diet which would induce polyneuritis.

He found that when the sick birds were fed Lloyd's preparation, made by adsorbing the vitamines from honey upon silicious earth, their recovery was not sufficient to warrant the conclusion that the honey contained any marked curative substance. However, when the birds were fed vitamines extracted from the wheat embryo they recovered very quickly.

In order to compare the ability of Yeast 51 to obtain the necessary vitamines from honey with that of pigeons, a culture medium was made with the same composition as Nägeli's solution with the exception of the amount of water and sugar. In Nägeli's solution 10 gm. of sugar were added for every 100 cc. of

TABLE IX.

Suspension No.	Tube No.	Gas produced after	
		5 days.	7 days.
		per cent	*per cent*
1	1	40	100
	2	45	100
	3	45	100
2	1	10	75
	2	5	95
	3	15	80
3	1	5	65
	2	0	70
	3	0	90

water. In the honey medium 25 cc. of strained honey were added to every 75 cc. of water. The salts were in the same proportion as in Nägeli's solution. This medium was sterilized as usual for 15 minutes at 15 pounds pressure in the autoclave. It was then inoculated with Yeast 51. Three suspensions of the yeast were used and triplicate inoculations of each made. After 16 days incubation none of the tubes showed any gas whatever. To determine whether the yeast cells were still alive and to determine whether there was a deficiency of the necessary vitamine in the medium, 0.5 cc. of sterile yeast extract was added to the inoculated medium after it had been incubated 16 days. Table X is a record of the results obtained.

It seems very certain from this table that the honey lacked the necessary vitamines for Yeast 51. The yeast extract used in this case was quite diluted; *i.e.*, it was not of the syrupy consistency described for the extract used in some of the preceding experiments.

Since it was found that several of the vitamine requirements of Yeast 51 are similar to those of animals, it seemed desirable to treat the vitamines in some way which is known to make them less valuable for animal feeding, to determine if by such treatment they might also be made less valuable for Yeast 51 and in this way further compare the vitamine needs of this yeast and animals. Chick and Hume (3) have stated that at about 120°C. the destruction of the antineuritic property of the wheat embryo

TABLE X.

Suspension No.	Tube No.	Gas after 16 days.	Gas after 4 days after adding 0.5 cc. of yeast extract.
		per cent	*per cent*
1	1	0	20
	2	0	50
	3	0	55
2	1	0	100
	2	0	100
	3	0	40
3	1	0	100
	2	0	65
	3	0	10

and of yeast extract is very rapid. It seemed of interest to test the effect of different degrees of heat for varying lengths of time on substances which Yeast 51 will ferment.

Yeast 51 grows very readily in cider with rapid fermentation. Hence it was decided to test the effect of heating the cider upon the rate or amount of gas production. Some of the cider was heated for 30 minutes at 100°C., some of it for 2 hours at 115°C. Triplicate inoculations were made with each suspension of the yeast. Table XI gives the percentage of gas produced after 3, 4, 7, and 11 days incubation of the cultures.

This table shows that the heating of the cider for 2 hours in

TABLE XI.

Suspension No.	Gas produced after			
	3 days.	4 days.	7 days.	11 days.
Cider heated 30 minutes at 100°C.				
	per cent	*per cent*	*per cent*	*per cent*
1	5	10	100	60
	0	2	40	65
	3	8	100	62
2	6	11	80	52
	3	5	100	100
	2	5	100	60
3	0	1	38	52
	0	20	40	93
	0	0	41	91
Cider heated 30 minutes at 115°C.				
1	0	5	100	50
	0	0	75	100
	0	0	100	55
2	0	0	55	70
	0	0	37	100
	0	0	48	95
3	0	0	18	100
	3	18	100	100
	0	4	43	100
Cider heated 2 hours at 115°C.				
1	0	0	90	98
	0	0	90	98
	0	0	100	100
2	0	0	85	100
	0	0	75	100
	0	0	35	100
3	0	0	100	100
	0	0	90	100
	0	0	84	100

an autoclave at 115°C. does not destroy its value as a medium for growth and fermentation of Yeast 51, but the table also shows very clearly that the time before fermentation begins is considerably increased. It did not seem desirable to test the fermentation of the yeast in the uncooked cider because of the many contaminating yeasts and other organisms which are present in fresh cider. However, in the cider which was heated only 30 minutes at 100°C. fermentation has begun in 3 days where the inoculation is as heavy as in Suspensions 1 and 2 while in the cider heated for 30 minutes at 115°C. fermentation begins about the 4th day and in that heated for 2 hours at 115°C. it begins some time after the 4th day. The maximum percentage of gas may be produced even if the cider has been heated for 2 hours at 115°C.

Some experiments reported above had shown that a very small amount of milk added to Nägeli's solution furnished the necessary substances for growth and fermentation with Yeast 51. To test the effect of heat on milk with reference to its stimulating effect on growth and fermentation with Yeast 51 one drop of raw or heated milk was added to each tube of Nägeli's solution. The milk was obtained under as nearly aseptic conditions as possible; *i.e.*, the milker milking directly into small sterile flasks which were then immediately plugged. Some of the milk was pasteurized at 63°C. for 30 minutes, some of it heated to 100°C. for 30 minutes, some steamer-sterilized by heating at 100°C. for 30 minutes on 3 successive days, some autoclaved for 15 minutes at 15 pounds, some autoclaved for 1 hour at 15 pounds, and some autoclaved for 2 hours at 15 pounds. Table XII gives the percentage of gas in tubes containing one drop of milk after 4 and 10 days incubation.

When 0.2 cc. of milk was added to tubes results as given in Table XIII were obtained.

These tables show that heating milk lessens its value as a stimulant for fermentation of Nägeli's solution with Yeast 51. It is a common dietary practice in infant feeding to supplement a diet of boiled milk with orange juice. In the decreased value of the heated cider and milk for Yeast 51, this organism appears again to be similar in its vitamine requirements to those of animals.

TABLE XII.

Suspension No.	Raw.		Pasteurized.		Boiled.		Steamer-sterilized at 100° 3 days.		Autoclaved at 15 pounds pressure.					
									15 min.		1 hr.		2 hrs.	
	per cent		*per cent*		*per cent*		*per cent*		*per cent*		*per cent*		*per cent*	
No. of days.	4	10	4	10	4	10	4	10	4	10	4	10	4	10
1	40	100	0	20	0	15	0	20	0	30	0	5	0	0
	45	100	0	10	0	5	0	40	0	35	0	0	0	0
	45	100	0	15	0	5	0	35	0	25	0	0	0	0
2	10	100	0	45	0	45	0	15	0	40	0	10	0	0
	5	100	0	20	0	30	0	20	0	45	0	15	0	0
	15	100	0	30	0	5	0	10	0	35	0	0	0	0
3	5	95	0	35	0	35	0	5	0	25	0	0	0	0
	0	100	0	40	0	50	0	15	0	40	0	0	0	0
	0	85	0	25	0	45	0	10	0	55	0	0	0	0

TABLE XIII.

Suspension No.	Steam-sterilized.		Autoclaved at 15 pounds pressure.			
			15 min.		2 hrs.	
	per cent		*per cent*		*per cent*	
No. of days.	3	5	3	5	3	5
1	55	100	50	100	0	20
	40	100	45	100	0	15
	45	100	50	95	0	5
2	25	100	45	80	0	15
	20	85	40	85	0	10
	35	100	35	90	0	20
3	15	100	15	75	0	5
	5	100	0	45	0	0
	20	100	20	80	0	5

SUMMARY.

The results of the experiments recorded in this paper show that both Pasteur and Wildier were right in their observations on the nutritive requirements of yeast. Pasteur had observed that yeasts would cause a medium composed of inorganic salts in solution and sugar to ferment. Wildier found that, in his studies, fermentation of such a medium occurred only if the amount of the inoculating material was considerable or if some organic matter was added to the medium. Wildier's studies in 1901 anticipated the later general interest in vitamines. He gave a special name "bios" to the substance which he thought was lacking in the inorganic culture medium. In my studies I have found that in the *Saccharomycetes* we have organisms which vary in their needs for traces of organic matter other than sugar in the medium. All the yeasts which I have used grow better and ferment more readily in a medium containing some small amounts of organic material. I have also found that some yeasts will grow in and ferment a solution of inorganic salts and sugar even when they are introduced in small amounts. In these points my observations agree with those of Pasteur.

I have also found that a certain yeast, which I have isolated from fermenting canned pears, will not grow in or ferment a solution of inorganic salts plus sugar unless introduced in very large amounts and even then the fermentation is not vigorous. For this yeast to produce fermentation some organic substance must be added to Nägeli's solution. The substances which when added to Nägeli's solution make it possible for Yeast 51 to cause fermentation are the substances which have been found to be rich in vitamines, especially water-soluble B. My work suggests that such yeasts as this one might be used to determine the presence of certain vitamines and possibly also that they might be used to determine quantitatively the amount of vitamines present in various substances. In these observations I have confirmed the work of Wildier.

The amount of organic material required is so small and the effect upon the growth and activity of the yeast is so striking that a marked similarity exists between these organic substances and the vitamines required for the normal development of ani-

mals. While some yeasts appear to be able to cause fermentation in a medium which contains little or no vitamines, Yeast 51 which I have described is not, nor is it capable of synthesizing its own vitamines.

I wish to acknowledge my indebtedness to Professor W. D. Frost for various helpful suggestions during the progress of the work here reported.

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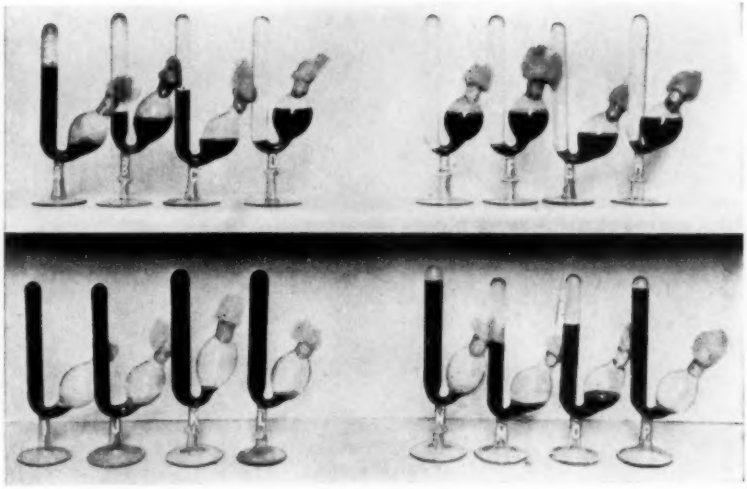
EXPLANATION OF PLATE.

All tubes were incubated at 28°C. for 4 days.

Tubes	A	and	B	inoculated	lightly,	Yeast	3 + 2	cc.	orange	peel extract.
"	C	"	D	"	"	"	5 + 2	"	"	"	"
"	E	"	F	"	heavily,	"	3 + 2	"	"	"	"
"	G	"	H	"	"	"	5 + 2	"	"	"	"
"	I	"	J	light	inoculation,	"	3,	no	orange	peel extract.
"	K	"	L	"	"	"	5,	"	"	"	"
"	N	"	M	heavy	"	"	3,	"	"	"	"
"	O	"	P	"	"	"	5,	"	"	"	"



(Bachmann: Vitamine requirements of yeasts.)

DETERMINATION OF AMMONIA IN BLOOD.

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(Received for publication, July 28, 1919.)

Under the title given above Morgulis and Jahr[1] have recently described a method involving the precipitation of the blood proteins with *m*-phosphoric acid and the extraction of the ammonia from the filtrate by means of permutit. The combination is a simple and alluring one and it has been tried a great many times in this laboratory (by myself and Dr. Denis) but was finally abandoned because of the numerous sources of error encountered. Every reagent used, including the permutit, must be absolutely free from ammonia and none of the reagents used can be purchased free from traces of ammonia large enough to materially influence the ammonia content of the blood.

All samples of potassium oxalate which we were able to obtain contained enough ammonia to double the amount present in blood. The presence of ammonia in oxalate can be demonstrated by direct Nesslerization, using 0.5 gm. of oxalate at a time. Ammonia-free potassium oxalate can be obtained by boiling a 5 per cent solution (2 liters) with 100 cc. of normal potassium hydroxide until a small volume (500 cc.) remains. The alkali is then neutralized with normal oxalic acid. The small amount of ammonia introduced with the oxalic acid is insignificant. By the addition of an equal volume of alcohol, previously treated with permutit, a considerable amount of substantially ammonia-free oxalate is obtained. We use regularly this grade of oxalate, but it is not necessary to do so except in connection with ammonia determinations.

All *m*-phosphoric acid preparations give color with Nessler's reagent. To get rid of this ammonia (and of the surplus water) proceed as follows:

Introduce into a previously washed and dried graphite crucible (capacity 600 cc.) 2 gm. of potassium nitrate and, on top of it,

[1] Morgulis, S., and Jahr, H. M., *J. Biol. Chem.*, 1919, xxxviii, 435.

200 gm. of glacial phosphoric acid. Heat over a radial Fletcher burner, very cautiously at first because of the excessive foaming. When the foaming has subsided skim off the dirt which floats on the surface. A silver spoon can be used for the skimming process if rapidly washed off in running water. Heat until all the water, 15 to 20 per cent by weight, is driven off and white fumes (P_2O_5) come off. Continue the heating at a high temperature for not less than 1 hour. The nitrous fumes come off early and also destroy the ammonia. Let cool until the melt is getting distinctly viscous and pour in shallow layers on tinned pans (pie plates). If the melt is too hot some of the tin plating comes off but this does no harm. When cool break up the pieces in a mortar as rapidly as possible. Transfer to bottles and stopper very tightly. The melted *m*-phosphoric acid cannot be poured on porcelain plates because it sticks fast and cannot be removed. The *m*-phosphoric acid prepared according to the directions given above is very much more effective than ordinary glacial phosphoric acid as a protein precipitant and it is free from ammonia.

Its preparation should perhaps have been described before, because much of the glacial phosphoric acid now sold is not an adequate protein precipitant and has been the cause of many erroneous non-protein nitrogen determinations. Since we have abandoned the use of it a full discussion of the subject has been omitted. It should be said, however, that the fused *m*-phosphoric does not possess the keeping quality of the commercial product.

The permutit used for minute traces of ammonia must be passed through an 80 mesh sieve, or better a 100 mesh sieve, yet must be free from dust; it must also be treated two or three times with alkali and Nessler's reagent to render it absolutely free from ammonia, and should then be washed with ammonia-free water, 2 per cent acetic, and again with water.

When these precautions are taken the amount of ammonia obtained from protein-free blood filtrates is practically nothing and only a greenish yellow color is obtained on Nesslerizing. The disturbing factors are certain amino-acid derivatives present in the blood filtrates and in part absorbed by the permutit.[2] Hiding this phenomenon by the addition of known amounts of ammonia as Morgulis and Jahr have done does not remedy this serious if not fatal source of error.

[2] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 91.

STUDIES OF ACIDOSIS.

XVI. DETERMINATIONS OF BICARBONATE IN THE BLOOD PLASMA OF DIFFERENT SPECIES BY THE TITRATION AND CARBON DIOXIDE CAPACITY METHODS.

By EDGAR STILLMAN.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, July 9, 1919.)

In a previous paper[1] Van Slyke, Stillman, and Cullen have described a method for titrating the bicarbonate content of the blood serum or plasma, and given results obtained with human and rabbit plasmas. In the present paper we give a more extended comparison of results obtained by the titration method with parallel results by the carbon dioxide capacity method. We have used plasmas from the blood of man, dogs, sheep, rabbits, and chickens.

In each case the bicarbonate has been determined before and after the addition of known amounts of standard acetic acid, as in the last experiment on page 174 of the former paper. 5 cc. portions of oxalate plasma were treated with water, or water plus 0.1 N acetic acid, making the total volume up to 6.5 cc. Portions were then taken for bicarbonate determinations by the carbon dioxide capacity method[2] and by the titration method. Neutral red was used as indicator.

In order to avoid the use of decimals, we have expressed the results in terms of millimolecular concentration (1 millimolecular = 0.001 molecular), rather than molecular.

In the first determination with each plasma no acid was added, but the 5 cc. of plasma were diluted to 6.5 cc. with water. Consequently in order to calculate the bicarbonate concentrations of

[1] Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1919, xxxviii, 167.

[2] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 317.

the undiluted normal plasmas, the values given are to be multiplied by $\frac{6.5}{5}$, or by 1.3. The values so calculated agreed well with those obtained by direct determinations on undiluted plasma.

Testing Standard 0.02 N NaOH for Carbonate.—The importance of using carbonate-free standard alkali was emphasized in the former paper. The solutions should be made up as there described, using only boiled water, be kept in paraffin-lined bottles, and be protected from atmospheric CO_2 by soda-lime tubes. They should be tested for carbonate as follows.

To 5 cc. of 0.02 N HCl in a 200 cc. round flask, add from a freshly filled burette about 4.8 cc. of the 0.02 N NaOH to be tested, with 0.3 cc. of neutral red solution. The mixture should be strongly acid to the indicator. The solution is rotated for 1 minute in the flask to permit the escape of CO_2, and is then transferred to a 50 cc. Erlenmeyer flask and titrated as in plasma analyses, the total amount of 0.02 N NaOH required to give the end-point being noted.

TABLE I.

Bicarbonate Determinations in Dog Plasma.

Plasma.		0.1 N acetic acid.	Water.	CO_2 bound as bicarbonate per 100 cc. of treated plasma.	Concentration of bicarbonate.		Fall in bicarbonate concentration.		Concentration of acetic acid added.
No.	Volume.				By CO_2 capacity.	By titration.	By CO_2 capacity.	By titration.	
	cc.	*cc.*	*cc.*	*cc.*	*millimol.*	*millimol.*	*millimol.*	*millimol.*	*millimol.*
I	5	0	1.5	41.4	18.5	16.6	0	0	0
	5	0.5	1.0	27.1	12.1	9.8	6.4	6.8	7.7
	5	1.0	0.5	2.6	1.2	3.2	15.3	13.4	15.4
II	5	0	1.5	48.1	21.5	20.7	0	0	0
	5	0.5	1.0	29.0	12.9	13.4	8.6	7.3	7.7
	5	1.0	0.5	6.1	2.7	5.8	18.8	14.9	15.4
III	5	0	1.5	38.5	17.2	15.1	0	0	0
	5	0.5	1.0	22.6	10.1	8.1	7.1	7.0	7.7
	5	1.0	0.5	8.5	3.7	1.2	13.5	13.9	15.4
IV	5	0	1.5	52.4	23.4	21.0	0	0	0
	5	0.5	1.0	34.0	15.1	13.8	8.3	7.2	7.7
	5	1.0	0.5	17.9	8.0	6.0	15.4	15.0	15.4

TABLE II.

Bicarbonate Determinations in Chicken Plasma.

Plasma.		0.1 N acetic acid.	Water.	CO_2 bound as bicarbonate per 100 cc. of treated plasma.	Concentration of bicarbonate.		Fall in bicarbonate concentration.		Concentration of acetic acid added.
No.	Volume.				By CO_2 capacity.	By titration.	By CO_2 capacity.	By titration.	
	cc.	*cc.*	*cc.*	*cc.*	*millimol.*	*millimol.*	*millimol.*	*millimol.*	*millimol.*
I	5	0	1.5	51.4	23.0	23.0	0	0	0
	5	0.5	1.0	32.8	14.6	15.6	8.4	7.4	7.7
II	5	0	1.5	39.9	17.8	18.2	0	0	0
	5	0.5	1.0	25.0	11.1	10.1	6.7	8.1	7.7
	5	1.0	0.5	7.5	3.3	3.2	14.5	15.0	15.4
III	5	0	1.5	33.4	14.9	14.1	0	0	0
	5	0.5	1.0	18.3	8.2	6.8	6.7	7.3	7.7
	5	1.0	0.5	0.0	0.0	0.8	14.9	13.3	15.4
IV	5	0	1.5	35.4	15.8	15.2	0	0	0
	5	0.5	1.0	20.8	9.8	8.5	6.0	6.7	7.7
	5	1.0	0.5	6.2	2.8	0.0	13.0	15.2	15.4

TABLE III.

Bicarbonate Determinations in Sheep Plasma.

Plasma.		0.1 N acetic acid.	Water.	CO_2 bound as bicarbonate per 100 cc. of treated plasma.	Concentration of bicarbonate.		Fall in bicarbonate concentration.		Concentration of acetic acid added.
No.	Volume.				By CO_2 capacity.	By titration.	By CO_2 capacity.	By titration.	
	cc.	*cc.*	*cc.*	*cc.*	*millimol.*	*millimol.*	*millimol.*	*millimol.*	*millimol.*
I	5	0.0	1.5	43.9	19.6	19.6	0	0	0
	5	0.5	1.0	25.9	11.5	11.7	8.1	7.9	7.7
	5	1.0	0.5	13.6	6.1	2.2	13.5	17.4	15.4
II	5	0.0	1.5	52.3	23.3	23.7	0	0	0
	5	0.5	1.0	35.4	15.8	16.6	7.5	7.1	7.7
	5	1.0	0.5	22.3	9.9	9.2	13.4	14.5	15.4
III	5	0.0	1.5	46.7	20.8	20.3	0	0	0
	5	0.5	1.0	28.9	12.9	11.6	7.9	8.7	7.7
	5	1.0	0.5	17.4	7.8	2.2	13.0	18.1	15.4
IV	5	0.0	1.5	42.9	19.6	17.7	0	0	0
	5	0.5	1.0	28.9	12.9	11.0	6.7	7.7	7.7
	5	1.0	0.5	14.8	6.6	1.8	13.0	15.9	15.4

TABLE IV.

Bicarbonate Determinations in Human Plasma.

Plasma.		0.1 N acetic acid.	Water.	CO_2 bound as bicarbonate per 100 cc. of treated plasma.	Concentration of bicarbonate.		Fall in bicarbonate concentration.		Concentration of acetic acid added.
No.	Volume.				By CO_2 capacity.	By titration.	By CO_2 capacity.	By titration.	
	cc.	*cc.*	*cc.*	*cc.*	*millimol.*	*millimol.*	*millimol.*	*millimol.*	*millimol.*
I	5	0.0	1.5	51.8	23.1	23.9	0	0	0
	5	0.5	1.0	36.9	16.5	14.4	6.6	9.5	7.7
	5	1.0	0.5	21.8	9.7	8.5	13.4	15.4	15.4
	5	1.5	0.0	9.6	0.4	1.7	22.7	22.2	23.1
II	5	0.0	1.5	52.2	23.3	24.1	0	0	0
	5	0.5	1.0	38.9	17.4	16.4	5.9	7.7	7.7
	5	1.0	0.5	24.4	10.9	9.9	12.4	14.2	15.4
	5	1.5	0.0	9.6	1.7	1.9	21.6	22.2	23.1
III	5	0.0	1.5	51.4	22.9	23.6	0	0	0
	5	0.5	1.0	36.8	16.4	16.3	6.5	7.3	7.7
	5	1.0	0.5	21.9	9.8	9.4	13.1	14.2	15.4
	5	1.5	0.0	9.6	1.7	1.9	21.2	21.7	23.1
IV	5	0.0	1.5	53.6	23.9	24.6	0	0	0
	5	0.5	1.0	38.2	17.0	17.2	6.9	7.4	7.7
	5	1.0	0.5	23.5	10.5	10.8	13.4	13.8	15.4
	5	1.5	0.0	12.1	5.4	3.3	18.5	21.3	23.1

A duplicate titration is performed in the same way, except that there is no agitation to remove carbon dioxide, the 0.02 N HCl plus 20 cc. of water being placed directly in the 50 cc. Erlenmeyer flask, and the 0.02 N NaOH being added with a minimum of stirring.

If there is no carbonate in the standard NaOH solution the two titrations give identical results. The difference should preferably not exceed 0.1 cc., and if it exceeds 0.2 cc. the alkali should not be used.

Tables I to IV show the parallel results obtained in determining the bicarbonate in the blood plasma of four different species by the titration and carbon dioxide capacity methods.

Comparison of Results by the Two Methods.—Results by titration agreed with those by CO_2 capacity as a rule within less than 2

millimolecular in plasma bicarbonate concentration, although in a few analyses this difference was exceeded. In the human plasmas the average difference was 0.8 millimolecular, corresponding to 1.7 volumes per cent of plasma CO_2. The results confirm the conclusions reached in the former paper, that the two methods may be used interchangeably for clinical and for many experimental purposes.

Results of practically the same degree of accuracy were obtained with plasmas from the different species examined; *viz.*, man, dog, rabbit, sheep, and chicken.

Comparison of Amount of Added Acid with Fall in Bicarbonate Concentration.—With both methods the amount of added acid was indicated by an approximately equivalent fall in plasma bicarbonate, the average difference between the calculated and observed changes being 1.4 millimolecular concentration by the CO_2 capacity method and 0.9 millimolecular by the titration.

CONCENTRATION OF AMMONIA IN BLOOD.

COMPARISON WITH CONCENTRATION OF AMMONIA IN DIFFERENT SECRETIONS AND TISSUES, ESPECIALLY MUSCLE TISSUE.

By K. L. GAD-ANDERSEN.

(From the Laboratory of Zoophysiology, Copenhagen University, Copenhagen, Denmark.)

(Received for publication, July 14, 1919.)

Sumner[1] has published a method of direct estimation of urea and ammonia in muscle. The method is a modification of the urease method, described by Marshall[2] and elaborated by Van Slyke and Cullen,[3] the only difference being that Sumner does not, like Marshall, grind the muscle with sand and thereupon extract the urea by means of alcohol, but only cuts the muscle into small pieces by letting it pass through a mincing machine several times; thereupon he weighs off a suitable amount, 2 to 3 gm., to which are added water and urease solution. After standing for about ½ hour all the urea will be diffused from the muscle and transformed into ammonia. If the ammonia is to be estimated, the manner of proceeding is the same; only anhydrous potassium carbonate is added directly and the ammonia is distilled off in the usual manner by a rapid current of air bubbling through. By this method Sumner has obtained some estimations of urea and ammonia in muscle, and he has found that the content of ammonia in muscle normally amounts to 12 to 15 mg. per 100 gm., and that a slight increase of the ammonia figure takes place when the muscle stands 4 hours before the ammonia is estimated.

Occasioned by the results at which Sumner has arrived by this method, I will give a preliminary brief statement of work I have done regarding the concentration of ammonia in blood, compared

[1] Sumner, J. B., *J. Biol. Chem.*, 1916, xxvii, 95.

[2] Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xv, 493.

[3] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211; 1916, xxiv, 117.

with the concentration of ammonia in different secretions and tissues, especially muscle tissue.

Like all other earlier investigators who have been engaged in the estimation of ammonia in muscle, Sumner finds that the amount of ammonia in muscle is many times larger (25 to 30) than that in blood. While the investigators of later years have shown that the concentration of ammonia in blood is very low and nearly constant,[4] the figure normally lying between 0.25 to 0.50 mg. in 100 gm. of blood, the values found for ammonia in muscle have varied very much, but are usually about 25 to 30 times higher than those found for blood. The fact is, however, that the concentration of ammonia in muscle and in blood is the same. That all earlier investigators have found higher values in muscle is due to the fact that after death a transformation of the urea of muscle into ammonia takes place, and the longer the time before estimation of ammonia is made, the higher will be the value for ammonia. If a sufficiently long time passes, all the urea will be transformed into ammonia, and this will usually be the case in the course of about 4 to 5 hours. The values which the different investigators have found for ammonia nitrogen really refer to urea nitrogen. In this manner the great variation in values arrived at for ammonia in muscle can be explained, as the results found partly depend upon how quickly the work of analysis proceeds, and partly upon the concentration of urea in that muscle in which the ammonia is to be estimated.

Recently a work regarding the relation of ammonia in the organism has been published in Danish by Iversen.[5] This author finds that he cannot obtain constant values for ammonia in muscle before 5 hours have elapsed. If the ammonia is estimated at an earlier moment, he finds lower results than if he waits the 5 hours. The estimation is obtained by grinding the muscle with sand and pouring over it a solution of uranyl chloride in weak hydrochloric acid in order to deposit proteins, and the mixture is left for 5 hours, during which time it is stirred now and again in order to distribute the ammonia. After the 5 hours have elapsed, the ammonia is estimated by means of a micro method, worked out

[4] Henriques, V., and Christiansen, E., *Biochem. Z.*, 1916, lxxviii, 165; 1917, lxxx, 297.

[5] Iversen, P., Medicin. Doktordisputatts, Copenhagen, 1919.

by the author. The author is of the opinion that since he cannot obtain identical values before such a long time has elapsed, the ammonia cannot have diffused from the muscle before 5 hours. This, of course is not correct, as Sumner's method plainly shows, but it is due to the fact that only after 5 hours have passed is the urea transformed into ammonia, and then the same values are obtained if he waits 8 or 12 hours before estimating the ammonia. What it is that causes the urea to be transformed into ammonia I shall for the present leave unsaid, but the process does not cease, even if, as Iversen does, uranyl chloride in hydrochloric acid is added, whereas it will cease as I have found if the muscle is put immediately into alcohol cooled to a temperature of 20°C. below zero.

With these circumstances in view, I have prepared a method of estimating ammonia in muscle, and the principle of this method is that the animal is stunned by a blow at the back of the head, whereupon the muscle is excised as quickly as possible and placed in a tared weighing glass with a suitable amount of 96 per cent alcohol which previously had been cooled to a temperature of 20°C. below zero. The weighing glass is again weighed and the increase in weight indicates the weight of the muscle. Usually I have taken about 3 to 5 gm. of muscle and ground it quickly with sand in a mortar, which is also kept at a temperature well below zero by means of a freezing mixture. The mass of ground muscle is covered with alcohol from the weighing glass and the mixture is thereupon transferred to a bottle and the ammonia removed by a current of air in the usual way. If urea as well as ammonia is to be estimated, the same manner of proceeding is followed, only the mass of ground muscle is filtered and the deposits are washed with about 50 cc. of alcohol. To the filtrate are furthermore added 10 cc. of water and 10 cc. of a solution of monophosphate of potassium (6 gm. to 1,000 gm. of water) which is necessary for binding the ammonia, and which also serves to increase the activity of the urease towards the urea. The mixture is poured over into a Petri dish which is placed in a drying box, where warm air is passed above and below the dish. This will cause the alcohol to evaporate. The heat is regulated in such a manner that the temperature in the dish does not exceed 25°, as at a temperature of 30° a transformation of urea into ammonia will take place.

When a suitable amount has evaporated, *i.e.* to about 20 cc., this is poured into a measuring cylinder. The Petri dish is then rinsed with water, which is also poured into the measuring cylinder. The fluid is thereupon divided into two equal portions and in the one portion ammonia is estimated, and in the other urea, the latter being performed by the method of Marshall.[6] By means of this method I have estimated ammonia and urea in muscle, heart, liver, and fat tissue, and I have found that the concentration of

TABLE I.

Estimation of Ammonia and Urea Nitrogen in Muscle per 100 cc. of Blood.

Ammonia N.		Urea N.		Total.	
Frog Muscle.					
At once.	2 hrs. later.	At once.	2 hrs. later.	At once.	2 hrs. later.
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0.8	5.0	9.3	5.3	10.1	10.3
0.6	0.6	9.6	9.6	10.2	10.2
Rat Muscle.					
	4 hrs. later.		4 hrs. later.		4 hrs. later.
0.4	8.4	10.4	2.6	10.8	11.0
0.5	0.5	*10.7	10.7	11.2	11.2
Dog Muscle.					
	5 hrs. later.		5 hrs. later.		5 hrs. later.
0.4	12.5	12.6	1.0	13.0	13.5
0.3	0.3	12.7	12.7	13.0	13.0

ammonia as well as the concentration of urea is the same as in blood.

The estimation of ammonia and urea in blood I have performed by a new micro method which will be published later. By means of the same method I have estimated ammonia in bile, spinal liquid, and aqueous humour and I have found the same concentrations as in blood.

[6] Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53.

In the table I have stated several values, which show that the concentration of ammonia is the same in muscle and blood, when the estimation is performed in the manner described above. The table also shows that when the muscle is left for some time, an increase of the concentration of ammonia takes place in such a manner that the sum of the urea nitrogen and the ammonia nitrogen is constant, whereas the figures vary mutually; thus the figure for ammonia nitrogen increases exactly as the figure for urea nitrogen decreases in a certain interval of time.

When Sumner states that Marshall and Davis,[6] who have made comparative estimations of the concentration of urea in blood and muscle, have not found the relatively large amounts of ammonia in muscle, then this is not remarkable as they have not fractioned the estimation. Had they done this, they would have found the remarkable relation of a lower concentration of urea in muscle than in blood, but on the other hand a higher concentration of ammonia in muscle than in blood, and that the sum of ammonia and urea in muscle was equal to the urea in blood.

SUMMARY.

A method for the estimation of ammonia in tissues is described. The concentration of ammonia is, in contrast to what all earlier investigators have found, the same in muscle and in blood. After death a transformation of urea into ammmonia takes place. Estimations of ammonia have been made on heart, liver, and fat tissue as well as on bile, spinal liquid, and aqueous humour, the concentration in all cases being the same as in blood.

To the Chief of the Laboratory, Professor A. Krogh, I herewith tender my thanks for the interest and the help he has rendered me in this work.

THE ACTION OF PTYALIN.

By HUGH McGUIGAN.

(From the Laboratory of Pharmacology, College of Medicine, University of Illinois, Chicago.)

(Received for publication, July 21, 1919.)

In 1885 Chittenden and Smith[1] studied the action of ptyalin on starch and found that a relation between the amount of sugar formed and the amount of ptyalin used existed only when the saliva was diluted 50 to 100 times. With these dilute solutions the speed of the reaction for a short period of time is proportional to the concentration of the enzyme. The figures quoted by these authors are scarcely adequate to establish definitely the relationship.

Their work indicates, as Lea[2] afterwards proved, that the products of digestion soon act as a disturbing factor. Chittenden and Smith found that when 53 per cent of the starch has been converted into sugar (calculated as dextrose) the action of ptyalin stops or reaches a balance. To determine the amount of sugar formed they used Fehling's solution and weighed the copper oxide formed. We have in the main confirmed this finding by the use of the Lewis-Benedict method; but we have also found that the balance point shifts with the volume of saliva used, and that it may go as high as 75 per cent of the substrate—calculated as dextrose.

The explanation usually given for the inhibiting action is that it is due to the mass action of maltose on starch and that the reaction is a reversible one, a balance being soon established.

$$(C_{12}H_{20}O_{10})_n + H_2O = C_{12}H_{22}O_{11}$$

[1] Chittenden, R. H., and Smith, H. E., *Tr. Connecticut Acad. Arts and Sc.*, 1885, vi, 343.

[2] Lea, A. S., *J. Physiol.*, 1890, xi, 226.

In addition to this Mathews[3] states that maltose and dextrose combine with the enzyme. We can find no experimental basis for either statement.

A modification of the method for the determination of the diastatic activity of blood by Meyers and Killian[4] based on the Lewis-Benedict method of blood sugar determination lends itself readily to the investigation of this problem. The unchanged starch does not interfere with the color reaction.

Methods.

Various modifications of the method have been used, and the following adopted as adequate. A starch solution was prepared from soluble starch (Merck). This was brought to 40°C. and set in an incubator at that temperature. Saliva was diluted 1:5 or 1:10, filtered, and set in an incubator until it attained the temperature of 40°C. A series of flasks was also heated in the incubator, varying amounts of the saliva were added to the flasks, and these filled with starch to the mark. Variations in the above concentrations were also used.

1 or 2 cc. samples of the digest were taken at stated intervals for analysis. 1 or 2 cc. of 0.1 per cent dextrose were used as a standard. The 1 per cent starch paste itself has a reducing action of about 0.015 to 0.025 per cent so that in a determination this reducing power must either be balanced or accounted for. With stronger solutions of starch the sugar content is very important and, if not corrected, often introduces a large error. The samples were boiled with 8 cc. of Benedict's picric solution and 2 cc. of 20 per cent Na_2CO_3 and made to a convenient volume before reading in the colorimeter.

RESULTS.

The following experiments in which analyses were made after ½, 1, 2½, and 5 hours show the action of ptyalin.

[3] Mathews, A. P., Physiological chemistry, New York, 1915, 333.

[4] Meyers, V. C., and Killian, J. A., *J. Biol. Chem.*, 1917, xxix, 179.

Experiment 1.—Into 100 cc. flasks the following volumes of 1:10 saliva were introduced and the flasks filled with 1 per cent starch:

Flask No.	cc.
1	5
2	4
3	3
4	2
5	1
6	0.5
7	0.25

Flask No.	Amount of sugar determined after							
	½ hour.		1 hour.		2½ hours.		5 hours.	
	Formed.	Per cc. of saliva.	Formed.	Per cc. of saliva.	Formed.	Per cc. of saliva.	Formed.	Per cc. of saliva.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	0.356	0.071	0.400	0.080	0.416	0.083	0.444	0.089
2	0.294	0.073	0.400	0.100	0.416	0.104	0.432	0.108
3	0.250	0.083	0.320	0.106	0.400	0.133	0.400	0.133
4	0.160	0.080	0.250	0.125	0.356	0.178	0.390	0.130
5	0.078	0.078	0.130	0.130	0.214	0.214	0.308	0.154
6	0.035	0.070	0.058	0.116	0.125	0.250	0.166	0.332
7	0.022	0.088	0.029	0.116	0.073	0.292	0.100	0.400

Experiment 2.—Experiment 1 was repeated using 5, 4, 2, 1, 0.5, and 0.25 cc. of 1:10 saliva with the following results:

Flask No.	Amount of sugar determined after							
	½ hour.		1 hour.		2 hours.		5½ hours.	
	Formed.	Per cc. of saliva.	Formed.	Per cc. of saliva.	Formed.	Per cc. of saliva.	Formed.	Per cc. of saliva.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	0.484	0.097	0.500	0.100	0.500	0.100	0.517	0.104
2	0.444	0.111	0.456	0.114	0.500	0.100	0.520	0.130
3	0.258	0.129	0.360	0.180	0.472	0.236	0.421	0.210
4	0.115	0.115	0.162	0.162	0.250	0.250	0.348	0.348
5	0.047	0.094	0.083	0.166	0.067	0.268	0.200	0.400
6	0.028	0.112	0.040	0.160	—	—	0.125	0.500

Note that for ½ hour in almost the whole series the amount of sugar formed is in proportion to the volume of saliva, but as time goes on, perhaps due to the interference of the digestive products,

this relationship is lost. After 5 hours there is scarcely any relation between the amount of saliva and the amount of sugar formed; but the amount of sugar formed tends to reach the point of equilibrium, which is higher the larger the volume of saliva.

Equilibrium Point in Ptyalin Digestion.

Chittenden and Smith state that when 53 per cent of the starch has been converted into sugar the reaction stops. Under the conditions of their experiment this figure is apparently correct, but it changes with the volume of saliva used until it reaches *about* 70 per cent when no more is formed, no matter how much ptyalin is added.

In this experiment we used 5 cc. of 1:10 saliva in 100 cc. of starch solution and digested the mixture for 18 hours at 40° to determine the influence of the concentration of starch on the amount of sugar formed, and also on the balance point.

Starch solution.	Sugar formed.	Starch converted into sugar.
per cent	*per cent*	*per cent*
1	0.532	53.2
2.5	1.333	53.2
5	2.400	48.0
10	3.640	36.4

No correction for the reducing power of the original starch was made in this case since this does not materially interfere with the point of equilibrium.

These results show that under the conditions of this experiment when 53.2 per cent of the weight of the starch used is converted into sugar an equilibrium or complete stoppage of digestion is established. We have found the same figures in other experiments, and they agree with those found by Chittenden and Smith who used a different method of investigation. With the stronger solutions of starch the figures are probably not correct since the method is most accurate in dilute sugar solutions. Apparently the smallest fraction of the digestion products exerts an inhibitory influence and when 10 per cent of the starch is digested it is sufficient to destroy the obvious relation of the amount of ptyalin

used and the sugar formed. For sugar solutions of this strength the Benedict method is not applicable unless great dilution of the sugar is made; then any slight error is multiplied many times.

When relatively large volumes of saliva are used the equilibrium figure is about 70 per cent. This, we think, is more nearly the correct figure than 53 per cent; *e.g.*, when equal volumes of 1 per cent starch paste and saliva diluted 1:10 with water were digested 15 hours at 40°C. the following figures were obtained.

Sample No.	*per cent*
1	58.8
2	60.4
3	60.4
4	60.4
Average.	60.0

In Nos. 1 and 2, and 3 and 4, the saliva was from different individuals.

When equal volumes of 2 per cent starch and 1:10 saliva were used the percentage of sugar found in 18 hours was 64.0, 62.4, 60.4, and 62.0 per cent; average, 62.2 per cent.

To test the effect of varying the amount of 2 per cent starch and 1:10 saliva an experiment was performed with results as given in Table I.

TABLE I.

Solution No.	Solution.	Starch.
		per cent
1	50 cc. starch + 100 cc. saliva	0.66
2	50 " " + 50 " "	1.00
3	50 " " + 25 " "	1.32
4	50 " " + 15 " "	1.44
5	50 " " + 10 " "	1.66
6	50 " " + 15 " "	1.82
7	50 " " + 2½ " "	1.90
8	50 " " + 1 " "	1.96
9	100 " " + 1 " "	1.98

Toluene was added and analyses were made in 4, 18, and 66 hours. The results are summarized in Table II.

To determine whether or not the ferment was used up in the process, 10 cc. of each of the above digests from Table I—after equilibrium was established—were taken and added to 100 cc. of

TABLE II.

Solution No.	Amount of starch digested in		
	4 hours.	18 hours.	66 hours.
	per cent	*per cent*	*per cent*
1	65.4	68.5	68.5
2	60.4	64.5	66.6
3	60.6	62.4	69.0
4	56.5	66.6	66.6 (?)
5	54.0	60.2	60.2
6	52.7	57.6	56.0
7	40.9	55.2	53.2
8	40.2	48.5	52.0
9	28.8	44.3	51.0

TABLE III.

Solution No.	Amount digested approximately.
	per cent
1	50
2	50
3	46
4	43
5	39
6	22
7	13
8	8
9	7

TABLE IV.

Solution No.	Starch digested after		
	20 hours.	44 hours.	6 days.
	per cent	*per cent*	*per cent*
1	70.0	65.5	62.4
2	70.4	69.6	64.0
3	70.0	69.2	73.0
4	74.0	73.2	71.1
5	66.5	69.0	64.0
6	65.9	67.6	50.2
7	65.2	66.1	65.7
8	63.2	65.8	63.7
9	34.8	36.0	42.5

fresh 2 per cent starch solution and again incubated 4 hours. It was then found that additional digestion as given in Table III occurred.

These figures show that when equilibrium was established, the ferment was still active.

This experiment was repeated with results as given in Table IV.

Since diastase may be kept in solution apparently unchanged for months, it may be that if we had left these solutions long enough they would all have reached the same concentration. This is hard to do owing to the decomposition of the sugar by bacteria, which toluene will not entirely prevent.

Influence of Concentration of Starch on Ptyalin Action.

Influence of the concentration of starch on the amount of sugar formed was determined by adding 1 cc. of 1:20 saliva to varying concentrations of starch. The results are shown in Table V.

TABLE V.

Solution No.	Concentration of 100 cc. of starch.	Sugar formed after		
		30 minutes.	60 minutes.	160 minutes.
	per cent	*per cent*	*per cent*	*per cent*
1	1	0.013	0.023	0.068
2	2	0.023	0.040	0.114
3	5	0.033	0.054	0.150
4	7.5	0.016	0.072	0.170
5	10	0.022	0.060	0.152

To 25 cc. each of the concentrations given in Table V 0.5 cc. of 1:20 saliva was added and, after digestion for 30 and 60 minutes, analyses showed results as given in Table VI. Sugar formed when corrected for sugar in starch (0.02 per cent).

TABLE VI.

Solution No.	Sugar formed after	
	30 minutes.	60 minutes.
	per cent	*per cent*
1	0.043	0.123
2	0.051	0.108
3	0.058	0.140
4	0.063	0.142
5	0.054	0.150

If we exclude the first of the series here there is little change due to concentration; any change there is might be due to the interference of the digestive products which exert their greatest influence where the percentage of digestion is highest. There is always slightly more sugar formed in the higher than in the lower concentrations of starch.

Relation of Volume of Substrate to Amount of Sugar Formed.

Varying volumes of 1 per cent starch solution containing 1 cc. of 1:10 saliva were allowed to digest, and when analyzed gave results as recorded in Table VII.

TABLE VII.

Solution No.	Volume of starch solution.	Sugar digestion after	
		30 minutes.	60 minutes.
	cc.	per cent	per cent
1	25	0.026	0.050
2	50	0.022	0.045
3	100	0.026	0.045
4	200	0.032	0.052
5	500	0.050	0.055
6	1,000	0.080 (?)	(?)

These results indicate that:

(1) The same volume of saliva in varying volumes of 1 per cent starch produces the same amount of sugar. In large volumes of starch great care must be observed since small errors are multiplied many times. Also a control starch must be run with each determination.

(2) The constancy of the result speaks in favor of a combination of enzyme and substrate.

Action of Dextrose and Maltose on Ptyalin.

To test the action of these sugars on ptyalin mixtures were prepared as given in Table VIII.

These mixtures were placed in an incubator at 40°C. and samples were taken for analyses after 18 hours. The amounts of the starch digested are given in Table IX.

This shows a definite retarding action of dextrose, but 260 per cent dextrose does not stop the ptyalin action, therefore the amount of sugar (70 per cent) which is formed when equilibrium

TABLE VIII.

Solution No.	5 per cent dextrose.	Saliva 1:5.	2 per cent starch.	Dextrose in the final solution.	Starch.	Sugar in relation to starch.
	cc.	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*
1	25	1	50	2.5	0.96	260
2	20	1	50	2	1.16	172
3	10	1	50	1	1.56	64
4	5	1	50	0.5	1.76	28
5	1	1	50	1	1.92	5
6	0.5	1	50	0.05	1.94	2.5
7	0	1	50	0	1.96	

TABLE IX.

Solution No.	Starch digested.
	per cent
1	16
2	26
3	49
4	50
5	64
6	64
7	65

TABLE X.

Solution No.	1:5 saliva.	4 per cent starch.	10 per cent dextrose.	Dextrose in final volume.
	cc.	*cc.*	*cc.*	*per cent*
1	5	12.5	32.5	6.5
2	5	12.5	25	5
3	5	12.5	10	2
4	5	12.5	5	1
5	5	12.5	0	0

is established cannot be an important factor in establishing equilibrium.

The action of dextrose was tested again in a second series of mixtures made up according to Table X.

In these mixtures the saliva is constant, starch in all cases is 1 per cent in the final mixture, and dextrose varies from 6.5 to 1 per cent, or 650 to 100 per cent of the starch used.

The action of maltose was tested in a similar series, 10 per cent maltose being used instead of 10 per cent dextrose.

In a third series a mixture of equal parts of 10 per cent maltose and 10 per cent dextrose was used; standards in each case corresponded to the added sugars.

These mixtures were allowed to digest for 18 hours and analyzed. After subtracting the added sugar the amounts of the starch digested were determined as recorded in Table XI.

These results show that both dextrose and maltose exert some inhibitory influence on digestion but, in relation to the volume of starch used, 100 per cent of sugar has very little influence, and

TABLE XI.

Solution No.	Dextrose.	Maltose.	Dextrose plus maltose.
	per cent	*per cent*	*per cent*
1	0	0	0
2	0	0	0
3	20	40	28
4	66	66	43
5	73	76	68

could not account for the stoppage of digestion at the equilibrium point; some of the other digestive products must be more important in this respect.

Does ptyalin initiate the reaction or merely hasten one naturally in process?

To test this I have kept a 1 per cent starch solution, covered with toluene, in the incubator at 40°C. for 14 days. The reducing power of the original solution was 0.030 per cent. After 14 days it was 0.035 per cent. This difference is within the limits of error of the method. In view of the doubt that is justifiable here, regarding the presence of a natural hydrolysis, and the enormous rapidity of the action when ptyalin is added, one must be biased to hold the opinion that ptyalin does not initiate the action.

DISCUSSION.

The method used is perhaps as accurate as any that has been applied to this problem. We do not think, however, that claim to a greater accuracy than 5 per cent can be made. With low sugar content the method as is well known is good. In such cases a variance of 5 per cent means very little; however, with higher concentrations any error may be multiplied many times in calculation. In all cases, instead of using a permanent standard color, it is better to have a standard of 0.1 per cent sugar and run the standard with each determination.

Regarding the chief cause of the establishment of equilibrium, we have reached no conclusion. It is not due to dextrose or maltose, since the addition of one or both of these sugars in the concentration in which they occur when equilibrium is established has very little influence on the course of digestion. It is due more to other products. Whether or not there is sufficient change in the hydrogen ion concentration to account for this we have not yet determined.

Enzymes are not generally supposed to affect the point of equilibrium and are not held to initiate a reaction, but to accelerate it only. The results recorded above lead us to doubt the basis of this belief at least with regard to ptyalin. The fact that the larger the amount of ptyalin used, the larger the quantity of sugar formed, leads us to believe that ptyalin exerts a force and does work in the digestion of the starch. It may be that with time the same amount of sugar would be formed in all cases, an assumption which we think throws the burden of proof on those who believe it.

That there is practically no difference in the amount of sugar formed in dilute and concentrated solutions of starch by the same volume of saliva favors the opinion that in the digestion the ptyalin unites with the starch, and when the starch molecule is converted into sugar the ptyalin is again free to unite with more starch. The fact that added maltose or dextrose disturbs digestion to but a slight degree shows that the ptyalin does not unite with these sugars.

CONCLUSIONS.

1. Ptyalin in dilute solutions acts on starch at a rate directly in proportion to the amount of ptyalin present (confirmation of Chittenden and Smith).

2. The products of digestion soon interfere with the normal digestion. These interfering products are not dextrose or maltose.

3. The point of equilibrium is reached when about 70 per cent of the starch, calculated as dextrose, is converted into sugar.

4. Practically the same amount of sugar is formed by the same amount of ptyalin in varying concentrations of starch solutions.

5. Evidence is presented in favor of the view that ptyalin unites with the starch during digestion, and exerts a force which causes hydrolysis.

THE MICRO DETERMINATION OF NITROGEN BY DIRECT NESSLERIZATION AND OF TOTAL SOLIDS, IN DROP QUANTITIES OF HUMAN BLOOD.

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(Received for publication, July 26, 1919.)

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1. INTRODUCTION.

The development of this technique arose from the necessity of making frequently repeated determinations of certain constituents on the blood of the same individual and from the desirability of increasing the utility of some methods of blood analysis by applying them to a few drops of blood which can be quickly and easily obtained. In the course of investigations in chemical pathology now in progress, with both hospital patients and normal persons, it was found impracticable to take blood from a vein of the same individual several times in one day or for days in succession when serial determinations were desirable. Experience demonstrated that it was not only practical from the chemical standpoint but also far more convenient and agreeable to the subject, to make use of drop quantities of blood taken from the

fingers or from the ear lobes. For a single sample of blood these considerations are of less importance than for serial determinations, but the latter are increasingly demanded by the needs of pathological and physiological investigation and of practical medicine, where progressive conditions are to be studied and treated.

The following methods permit the estimation of nitrogen in a few drops of blood or similar nitrogenous liquid with an accuracy of a few thousandths of a milligram. They are characterized by the use of a fixed standard for colorimetric measurement instead of making a fresh standard for each estimation. We have also found a simple adaptation of this procedure easy to apply to muscle and similar tissue. Results which have been obtained on certain pathological problems will be subsequently published.

2. Collection and Measurement of Blood.

The principle here followed is that a few drops of blood can be accurately handled by distributing it in an easily measurable quantity of liquid medium and by ascertaining its total quantity in the first instance by *weighing*, making subsequent divisions by volumetric measurement. Human blood is hence obtained by drops from the fingers or from the ear lobes. Larger quantities than are needed for these methods can also be obtained in this way. For making the puncture we have found the use of a spring lance, which regulates the depth of the puncture according to the thickness of the skin at the selected location, so advantageous that we have used it exclusively. We have found the application of a layer of eucalyptol to the skin, previous to the puncture, very useful to prevent or delay the clotting and adhesion of the blood, which interferes with the free falling of the drops from the puncture. For reasons pertaining to another problem we have used a 0.5 per cent NaF solution as a very satisfactory collection medium for the blood.

When it is permissible to make the determinations on successive drops from the same puncture, two tubes, *A* and *B* each charged with 3 cc. of medium, are used. For total nitrogen about 2 drops are collected in *A* and diluted with the collection medium to about 10 mg. per cc. and for a single determination of non-protein nitrogen about 8 drops are collected in *B*. For total solids about 5 drops are collected in the test-tube *C* charged as described below.

When the determinations must be made on the same composite sample of blood we proceed as follows. One or more graduated centrifuge tubes, or test-tubes, best marked at 8.5 cc., are each closed with a soft rubber stopper or good cork, carrying a loop of small copper wire, whereby the charged tube can be suspended on the arm of the analytical balance and weighed. The blood is allowed to drop from the puncture directly into the liquid medium and the number of drops is counted. The minimum useful number of drops for all three determinations is 15 and the maximum number required even for duplication of the nitrogens is 30 to 36 drops. If many drops of blood are collected in one tube it should be gently tapped to distribute the blood as it drops into the liquid. The tube is at once closed and gently inverted a few times, or better rotated (never shaken to foam) in order to mix the contents. The second weight is then taken in order to ascertain by difference the weight of the blood. The average weight of a drop of blood obtained as above has been found to be about 35 mg. The entire quantity of liquid should next be made to a definite volume of about 8.5 cc. by running more of the same medium on the surface of the mixed blood by means of a pipette held against the inner wall of the graduated tube. The liquids are thus prevented from mixing and the total final volume of the blood dilution can be read off at the lower edge of the meniscus of the clear and colorless liquid of the upper layer. To save time in the subsequent evaporation of water it is desirable to maintain a small volume. When all the determinations are to be made on the same composite sample of blood drops, and when the two nitrogen determinations are also to be duplicated, we have found about 8.5 cc., carrying a maximum of about 30 to 35 drops of blood, to be abundantly adequate, even to include a determination of total solids. 1 cc. of this volume is accurately pipetted into a graduated cylinder and diluted with the collection medium to such a volume that the liquid will represent about 10 mg. of blood per cc. which is the amount of blood to be used for each determination of total nitrogen. Duplicates on total nitrogen are made from this same dilution. If a single determination of solids is desired 2 cc. of the remaining volume may be removed with a pipette to a test-tube charged as described below. There thus remains a little more than 5 cc. of diluted blood which can be used for non-protein nitrogen by either two determinations of 2 cc. each or one of 5 cc., depending on whether about 30 or about 15 drops of blood have been collected.

3. Determination of Total Solids.

The principle of this process consists in drying the blood mixed with a suspension talcum powder which prevents its caking to a mass that would retain moisture beneath its surface either locally or as a whole. Pure unscented talcum powder by reason of its physical properties seems to be excellently adapted to overcome the

difficulties in drying highly albuminous or colloidal materials whether liquid or solid. For receiving a previously made dilution of blood the test-tube is charged and weighed with a few decigrams of pure dry talcum powder. 1 cc. of the diluted blood (containing known weights of NaF and of blood) is run into the tube with a pipette and the contents are mixed. When blood is to be dropped directly from the puncture into the tube the latter is weighed with a few decigrams of dry talcum powder containing 10 per cent of dry NaF. A supply of the dry, mixed powder for this purpose is kept in a desiccator. After the tube thus prepared has received several drops of blood just enough distilled water is added to make a thick suspension. In either case after the blood has been introduced, the tubes are to be rolled without loss so as to distribute the talcum as much as possible over the inner surface of the tube. The tube is now laid in the drying oven in a very nearly horizontal position so as to expose the contents over a relatively large surface for evaporation. Under these conditions we have found a temperature of about 75°C. (purposely selected low) entirely satisfactory. After completion of the drying and cooling in a desiccator the second weighing is made in order to ascertain the weight of the blood solids by difference. Further drying and weighing have shown a very satisfactory constancy of the results.

4. Preparation of Blood for Non-Protein Nitrogen.

To one volume of diluted blood on which the non-protein nitrogen is to be determined we add two volumes of a fresh solution of 20 per cent trichloracetic acid and place a glass rod in the test-tube for stirring and transferring the contents. Let stand 15 to 30 minutes and with the aid of the rod transfer to a small conical glass funnel carrying a 55 mm. folded filter paper (*e.g.* Whatman No. 2) which has been wet with some of the trichloracetic acid.[1] When the liquid has run through wash the precipitate on the filter twice with 2 cc. portions each of the same trichloracetic acid solution, which portions are first placed in the original test-tube and then transferred by the rod. With a high concentration of the acid, precipitation and filtration proceed promptly but with too low a concentration, filtration becomes very slow. We have also found that the filtration can be very well accomplished with or without the use of a filter

[1] On rapid filtration see Whitcomb, L. W., *Chem. Anal.*, 1919, No. 28, 25 and references.

pump by the use of an asbestos filter-bed made on a number of small quartz pebbles placed in the neck of a 1 to 2 inch funnel. The filtrate is now ready for evaporation and digestion and is quantitatively transferred with the help of a small quantity of water to the digestion flask.

5. Reaction Liquid for the Kjeldahl Digestion.

An Erlenmeyer flask in which, as described below, the digestion is to be conducted, may contain 1 cc. of diluted blood for total nitrogen or from 12 to 20 cc. of trichloracetic filtrate for non-protein nitrogen. Both liquids are treated with the same reagents and subjected to the same subsequent processes. To each of these blood liquids are added 1 cc. of concentrated H_2SO_4, 4 drops of a saturated solution at room temperature of Na_2SO_4, and 2 drops of 10 per cent $CuSO_4.5H_2O$ solution. Drops are to be measured from glass tubing of 3 mm. internal diameter. To prevent or minimize bumping we use quartz pebbles as recommended by Folin. The form, dimensions, and inclined position of the Erlenmeyer flasks used for digestion adequately prevent loss from such bumping as may occur before the liquid boils, if six pebbles are placed in each flask and the heat is somewhat moderated during the first half minute. Powdered material from which the liquid cannot easily be removed by decantation or whose suspension would cause a turbidity that would interfere with the subsequent colorimetric reading must not be used.

6. Digestion of Blood in the Kjeldahl Process.

Experience has shown that this operation is the most critical one of the entire determination as it subjects the operator to greater liability of unobserved error than any other. We have made control tests on the repeated digestion of small amounts of nitrogen in standard ammonium sulfate solutions, under varying conditions of time, of heating power, and of temperature and concentration of digestion liquid. Losses of nitrogen, in amounts not permissible for this method, were easily incurred with microburners as well as with the larger Bunsen burners, which evidently were connected with both the shape and the size of the container and especially the heating power in relation to these. It is a fact, far from sufficiently recognized, that nitrogen is easily lost from a heated, concentrated H_2SO_4 solution of $(NH_4)_2SO_4$ also containing salts to raise the boiling point, even when, as in the present method there is a strong excess of H_2SO_4 intended to prevent or minimize this risk. The conditions of digestion should be repeatedly tested to demonstrate by control tests on reagents that 0.35 mg.

of standard nitrogen in ammonium sulfate containing a trace of pure sucrose, can be quantitatively recovered, after subjection to the digestion process exactly under the mechanical and heating conditions that are to be constantly used in the case of blood. We have tested and adopted the following standard conditions.

Erlenmeyer flasks of about 200 cc. capacity are used for evaporation, digestion, and subsequent operations. We prefer these flasks to either small Kjeldahl flasks or to large and long test-tubes, because, when used as described below, the large surface secured from a given bulk of liquid and the comparatively short length and wide neck of these flasks permit rapid evaporation of large volumes of liquid. This is a very advantageous condition for some work but especially so for non-protein nitrogen filtrates and for the evaporation of trichloracetic acid. Furthermore the angle between the base and the wall of these flasks is excellently adapted to the subsequent digestion in such small volume as 1 cc. or less of concentrated H_2SO_4. When these processes are conducted as described very rapid evaporations and digestions are made without any loss of nitrogen. The flasks we used were made of Pyrex glass with rather thick wall and they measured about 12.5 cm. vertical length, 8 cm. outside diameter of base, and 2.5 cm. diameter of neck. The flask is placed in an inclined position of about 45°, supported by the angle at its base on a clay triangle, which is carried by a ring clamped to a stand. The straight neck of the flask rests against a 10 inch rod of glass tubing about 1 cm. in diameter, which is clamped to the same stand at the proper height for the inclination of the flask. A small rubber band slipped loosely over both glass rod and neck of flask holds the latter in position. A microburner of the customary size is clamped to the same stand so that it can be swung out to one side when the digestion is finished, thus leaving the flask in position to cool. The distance between the upper edge of the burner and the angle of the flask should be short; *e.g.*, about 3 cm. Exactly the same results as with a microburner can be obtained, but with less convenience, with a good, perfectly regulable Bunsen burner of the ordinary large type, similarly clamped in position, provided the burner can be so regulated as to work well with a very short as well as a longer flame.

The most important point in the management of a digestion relates to the process of heating. In our selection of apparatus and arrangement of conditions we designed that the liquid to be evaporated shall be vigorously boiled but without loss of material, by the use of a flame sufficiently large to expel the excess liquid very rapidly. We have demonstrated that 10 cc. of liquid can be safely removed from the above described apparatus in 5 minutes from the beginning of heating. When the digest has become concentrated in the angle of the flask and as white fumes begin to fill it, the flame should be quickly diminished so that merely its tip touches the flask, no more heat than enough to maintain boiling being applied. The operator should always bear in mind the great liability

to error by overheating the digest at this stage. The appearance of white fumes is also the signal for covering the neck of the flask with an inverted crucible which encloses or condenses the fumes, some liquid dripping from the edge of the crucible but not falling on the hot flask owing to its inclined position. By this procedure digestions can be made anywhere in the laboratory without precaution against fumes. As in the present method the total nitrogens are already in a volume of about 2 cc., one of which is concentrated H_2SO_4, the period of evaporation is very brief, the entire process being completed in 8 to 12 minutes from the beginning of heating. In the case of non-protein nitrogens and of control digestions on standard $(NH_4)_2SO_4$ (7 cc. = 0.35 mg. of N) the initial volume will vary from 10 to 20 cc.

7. *Direct Nesslerization of Kjeldahl Digests.*

After the digestion liquid has somewhat cooled 15 cc. of distilled water are added, the whole is cooled under the tap, and then also 2 cc. of 5 per cent Rochelle salt are added. In order to prevent loss of ammonia from the heat of neutralization, addition of alkali and cooling under the tap are done stepwise. Hence the acid is to be first partially neutralized, in the same flask, by the addition of 10 cc. of approximately 10 per cent NaOH and the liquid is again cooled. Rotation of the contents greatly facilitates the cooling. Neutralization is now completed by the further addition of 10 per cent NaOH delivered from a burette, until a clear, dark blue color, due to the Rochelle salt solution of the $CuSO_4$, has become plainly evident, thus leaving the reaction very slightly alkaline. 5 cc. of the Folin-Denis *concentrated*[2] Nessler solution are delivered with a pipette into a wide-necked (CO_2) flask or beaker.

At this point it is necessary to note the fact, which was first observed by the use of the constant color standard described below, that the Nessler reaction is extremely sensitive to conditions of temperature, so that colorimetric differences of 1 to 3 mm. or more may be due to this cause alone. Agreement of duplicates or constancy of results on the same material (*e.g.* 0.35 mg. of standard N) may be entirely vitiated. For practical purposes and saving of time, uniformity of conditions may best be secured by long enough cooling under the tap of both the unknown, prepared as above, and the 5 cc. of concentrated Nessler solution, completely to reach the temperature of the tap water, when the latter is not more than 5° below room temperature. In this case the colorimeter cup should

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 479. The Nessler solution used throughout this work was made by mixing 300 cc. of the double iodide solution with 200 cc. of 10 per cent sodium hydroxide, but the further addition of an equal volume of water as directed by Folin and Denis was omitted in order to use as small a volume as possible; *i.e.*, 5 cc. of the concentrated Nessler solution.

be likewise cooled with the tap water. By another procedure the liquids, stoppered in their respective flasks, are allowed, after cooling, to reach room temperature before mixing, but this will require more time than the preceding but is also more accurate and uniform.

In any case the content of the Erlenmeyer flask is now rapidly poured into the Nessler solution, and not *vice versa*. By this procedure the Nessler solution, at no instant of time, ever falls below its ultimate concentration, as it would do in the reverse procedure, and quantitative uniformity of color and perfect clarity of Nesslerized liquids result. The liquid is now transferred to a measuring cylinder, made to a volume of 50 cc. with distilled water, and returned to the flask or beaker for the purpose of mixing. The depth of color of this Nesslerized digest is now to be immediately measured in terms of a constant color standard by means of the colorimeter. The liquids thus Nesslerized will keep for many hours or even a day but examination with the colorimeter against a constant color standard will show that they undergo easily measurable changes in depth of color and hence should be read immediately or at a convenient fixed period of time after Nesslerization.

8. Composition and Use of a Fixed Color Standard.

In a previous, unpublished investigation also dealing with drop quantities of human blood we learned the superiority of a constant inorganic color standard for all work of this nature. The solutions of colorimetric methods almost invariably undergo rapid change in intensity when measured against a fixed color standard, though such change may be invisible to the unaided eye. The use of a constant standard is likely to prove illuminating to anyone who has always made the customary assumption that his standard and his unknown are strictly comparable. The fixed standard brings to light previously unsuspected peculiarities and variabilities of methods. In any case the fixed standard has a very evident advantage in the saving of time as well as in accuracy over the customary procedure of making a new standard for each determination. In order to work within very small limits of error with the amounts of material here used we found it essential to observe in detail the described method of dealing with the colorimetric problem. These conditions were not arbitrarily chosen but were forced by the requirements of constancy and accuracy. The two principal factors involved are first, the composition of the color standard, and second, the method of using colorimetrically different lengths of the same color standard for

all values of unknowns falling within its range. *In other words we match by varying the standard against a fixed length of column of the different unknowns and not vice versa as is customary.* By selecting a sufficiently long column (30.0 mm.) for unknowns of small nitrogen content, we intensify both the color and the colloidal density, as seen in the optical field, to be matched by the single fixed standard and it thus becomes possible to measure very small amounts of nitrogen with certainty. The composition of the color standard that is to be used as a common yard stick against all unknowns must be such as to permit good matching throughout the necessary range of concentrations, which for these blood analyses always lies well within the limits of 0.15 to 0.50 mg. of N. For other purposes the conditions can be adjusted to larger amounts. The composition used must also be highly sensitive; *i.e.*, a change of 1 mm. on the color scale should represent a very small quantity in mg. of N as is well illustrated by the data in Table I. We abandoned numerous compositions which matched well at some one point only or region of the scale but which lacked sufficient sensitivity. The following solution was found excellently adapted to match the above Nesslerized blood digests as well as Nesslerized liquids from pure $(NH_4)_2SO_4$. A solution of ferric ammonium sulfate (of C. P. grade for analysis) is made containing 8 gm. per 100 cc. and another solution of picric acid, containing 1 gm. of the air-dry acid per 100 cc. To 75 cc. of the fresh ferric solution are added 2 cc. of the picric acid and the volume is then completed to 100 cc. with distilled water; or to 6 gm. of ferric ammonium sulfate dissolved in about 75 cc. of cold distilled water, 2 cc. of the 1 per cent picric acid solution are added, and the volume is then made to 100 cc. with distilled water. This latter procedure is preferable, as a stock 8 per cent solution of the ferric sulfate undergoes some precipitation after a time. The mixed color standard remains perfectly clear and of constant intensity of color from several days to a week or longer until some precipitation occurs, when a fresh solution should be made. We have had no difficulty in obtaining the same color intensity in different preparations of this color standard and the method of evaluation described below makes some variation immaterial even for quantitative accuracy. Old and new solutions are easily compared with the colorimeter by measuring 30.0 mm. columns of them against

a single standard as is described below for Nesslerized solutions. The reflecting mirror of the Duboscq colorimeter should always be covered with white filter paper (*e.g.* Whatman No. 1) the dull surface of which gives excellent results in matching with the color standard and the Nessler liquids. Color standard and Nesslerized solutions are always to be placed in the same respective cups and positions, preferably the former left and the latter right, as the observer faces the back of the Duboscq colorimeter. Experimentation has shown that the Nesslerized solution should always be set at a column of 30.0 mm. when it is to be measured in terms of the above described standard. Preliminary variations of the column length of the color standard show a green shade when too short, a red when too long, and the best possible matching for color is to be made between these extreme limits. Matching here means finding, by means of color and of colloidal density, a definite median position on the color standard, that can be reproduced with certainty by means of the same concentration of the standard nitrogen, and not necessarily perfect identity of color in the two half fields. For this quantitative reason also the matching is always completed by very short forward movements *in the direction of the lengthening column*, the developing red and increasing darkening of the right optical field (from the left hand cup) serving as an effectual stop. The observer must learn to distinguish between matching for shade of color and matching for illumination or density of the field, which latter is due to the colloidal state of both standard and Nesslerized liquid. For amounts of nitrogen less than 0.20 mg. it is necessary to match by the comparative densities of the two optical fields, instead of by color and density both, as for larger amounts. After a little experience matching with this color standard is done with ease and certainty.

9. Evaluation of Colorimetric Readings.

In order to ascertain the correct method of evaluation of the colorimetric readings found with a single fixed color standard, it is necessary to determine the quantitative relation that exists between the mg. of N, when developed by the standard procedure described and the mm. of column length. Numerous determinations under a variety of conditions, of the colorimetric powers of

0.35 mg. of N (=7 cc. of standard ammonium sulfate) as a basal value showed that the depth of color produced is much affected by the temperature at which the Nessler reaction occurs, all other factors being held invariable. We also found that the tabular standard values in mm. of color standard for 0.15, 0.20, 0.25, 0.30, 0.35, 0.45 mg. of N all fluctuated in harmony with the basal value

TABLE I.

Determination and Calculation of Nitrogen. Standard $(NH_4)_2SO_4$, Nesslerized without Digestion.

No. of determination.	Standard N.	S Color standard.	Interval for 0.35 mg. of N.	R Rate.	B Basal value.	Found N.	Error.
	mg.	mm.	mm.	$\frac{mg.}{mm.}$	mm.	mg.	mg.
Series I.							
1	0.15	17.5	20.4	0.0098	37.9	0.148	−0.002
2	0.25	27.3	10.6	0.0094	37.9	0.245	−0.005
3	0.35	37.9	0	0	37.9		
4	0.45	44.2	6.3	0.0159	37.9	0.450	0
Series II.							
5	0.15	16.4	19.7	0.0101	36.1	0.155	+0.005
6	0.35	36.1	0	0	36.1		
Series III.							
7	0.15	12.6	20.9	0.0096	33.5	0.143	−0.007
8	0.35	33.5	0	0	33.5		
Series IV.							
9	0.20	20.6	13.8	0.0109	34.4	0.213	+0.013
10	0.25	24.9	9.5	0.0105	34.4	0.256	+0.006
11	0.30	38.8	5.6	0.0089	34.4	0.295	−0.005
12	0.35	34.4	0	0	34.4		
13	0.45	40.5	6.1	0.0164	34.4	0.447	−0.003
Series V.							
14	0.35	33.3	0	0	33.3		
15	0.45	39.8	6.5	0.0154	33.3	0.453	+0.003

Average value of $R = 0.0099$ for $S < B$.
" " " $R = 0.0159$ " $S > B$.

for 0.35 mg. of N, in such manner that while the absolute values might increase or diminish several mm., the *differences* in mm. or the *intervals* for any two tabular values of mg. of N remained constant within the limits of experimental error. If the mg. of N are plotted as ordinates and the corresponding mm. of color standard as abscissæ, using one square of the plot to represent 0.01 mg. of N vertically and 1.0 mm. of color standard horizontally, the curves

obtained will always be of the same form and each curve will run *parallel to itself* when the basal value for 0.35 mg. of N varies. In other words the curves move 1 or more mm. to the right or to the left with alteration in their absolute values in mm. but without change in the number of mm. expressing the relation between any two amounts of N. The plotted curves or a comparison of the quantitative values of the intervals from 0.15 to 0.35 mg. of N and from 0.35 to 0.45 mg. of N shows that, for accurate evaluation of colorimetric readings, it is not permissible to substitute a single straight line for the experimentally ascertained curve. This evaluation can be easily and quickly made if the rate of change R, in mg. per mm. along the curve, is known in both directions from B, the basal value in mm. for 0.35 mg. of N. Let S = mm. of color standard for y mg. of N. Then when $S<B$, $y=0.35-R\ (B-S)$ and when $S>B$, $y=0.35+R\ (S-B)$. We thus adjust the evaluation of the colorimetric readings to a base which is determined with ease and rapidity and whose numerical variation is subject to the same analytical conditions as are the unknowns. These relations are illustrated in Table I. For ordinary purposes the given value of R may be accepted but for critical work the operator should make his own determination on the series 0.20 − 0.35 − 0.45 mg. of N.

10. Conditions of Accuracy of the Method.

These conditions should be considered from two points of view, first, the limit of error, *i.e.* the variability on repetition, and second, the comparative or absolute accuracy as measured by a known, correct standard. On the former point we have found that repetitions on the same material give results that fluctuate only a few 0.1 mm. of color standard, being essentially identical. For the latter standard we have adopted the micro-Kjeldahl distillation process applied with suitable apparatus to the same small amounts of nitrogen-containing material as in the method described above for direct Nesslerization. The distillates were collected in 5 cc. of 0.25 N H_2SO_4 and then Nesslerized in a final volume of 50 cc. and read against a 0.35 mg. of nitrogen standard Nesslerized in exactly the same way as the distillates and set at 30.0 mm.

Experiment.—Required to compare by direct Nesslerization and by distillation the amounts of N found in 1 cc. of diluted blood (= 10.6 mg. of blood) which had undergone slight decomposition.

(1) Found basal value, $B = 34.6$ mm.

(2) Directly Nesslerized digest by color standard, $S = 27.8$ mm. $\therefore$ Nesslerized $N = 0.350 - 0.0100\ (34.6 - 27.8) = 0.282$ mg. of N.

(3) To test the quantitative comparability of Nesslerized blood under the conditions of this method and of standard $(NH_4)_2SO_4 = 0.05$ mg. per cc., 5.6 cc. = 0.028 mg. of N, were taken from a burette and Nesslerized as formerly described.

Found by color standard, $S = 27.7$ mm. cf. $S = 27.8$ mm. in (2).

(4) Distilled the digest of 1 cc. of blood and treated as described above.

Distilled N read 36.8 mm. $= \frac{300}{368} \times 0.35 = 0.283$ mg. of N cf. 0.282 mg. of N in (2).

TABLE II.

Comparison of Total Nitrogen in Human Blood by Direct Nesslerization and by Distillation.

No. of determination.	Process.	Blood.	Color standard.	Total N.		Difference.
		mg.	*mm.*	*mg.*	*per cent*	*per cent*
Series I.						
1	Direct Nesslerization.	9.8	33.7	0.330	3.37	
2	" "	9.8	32.4	0.318	3.24	
1–2	" "	9.8	33.1	0.324	3.31	+0.14
3	Distillation.	9.8	34.5	0.304	3.10	
4	"	9.8	33.0	0.318	3.24	
3–4	"	9.8	33.8	0.311	3.17	
Series II.						
5	Direct Nesslerization.	10.0	37.6	0.327	3.27	
6	" "	10.0	39.0	0.334	3.34	
5–6	" "	10.0	38.3	0.331	3.31	−0.07
7	Distillation.	10.0	31.0	0.338	3.38	
Series III.						
8	Direct Nesslerization.	13.2	45.8	0.377	2.87	+0.05
9	Distillation.	13.2	27.6	0.372	2.82	
Series IV.						
10	Direct Nesslerization.	8.5	28.7	0.293	3.45	+0.09
11	Distillation.	8.5	36.6	0.287	3.37	

These microdistillations are very satisfactory and they are surpassed only in rapidity and convenience by Nesslerization. The results shown in Table II were obtained by an assistant who had recently learned this technique. We have compared, with entirely satisfactory results, this method of direct Nesslerization with that of distillation, on both the same specimen of human blood and on standard solution of pure ammonium sulfate, both with and without the digestion procedure described above.

11. SUMMARY.

A method has been described for the determination of total and non-protein nitrogen and of total solids in fifteen to thirty drop quantities of human blood so that frequent or serial determinations at short intervals can be conveniently made on the same individual.

The use of a single fixed color standard has been developed for the measurement of the Nesslerized nitrogen solutions.

The conditions of accuracy have been tested and the quantitative evaluation of the colorimetric results has been adjusted to variations of analytical conditions so that the variability and certainty of the results lie within a few thousandths of a milligram of nitrogen.

A procedure for determining the dry weight of a few drops of blood has been described which obviates incomplete or unequal drying.

RELATION OF PLANT CAROTINOIDS TO GROWTH, FECUNDITY, AND REPRODUCTION OF FOWLS.*

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PLATE 2.

(Received for publication, July 9, 1919.)

Schunck,[1] and Willstätter and Escher[2] have established the chemical identity of the natural yellow pigment of the yolk of hen eggs with one of the xanthophyll group of plant carotinoids. One of us[3] has studied especially the physiological relation between the plant carotinoids, particularly xanthophyll, and the lipochrome of the blood serum, body fat, and egg yolk of the hen. It has been shown that the amount of xanthophyll in the egg yolk and blood serum varies with the amount of xanthophyll in the food and that the color of the body fat deposited during fattening is determined by the proportion of xanthophyll which the ration carries. The further especially interesting fact was established that fowls make relatively little, if any, use of the carotin fraction of the plant carotinoids for the pigmentation of the adipose tissue and egg yolk. Quite the opposite has been shown to be the case for the cow,[4] and the horse,[5] which resorb the carotin almost exclusively. The adipose tissue, milk fat, and blood serum of sheep and swine, however, are entirely devoid of either group of carotinoids. No doubt this is true also for such species as the rabbit, dog, cat, etc., whose adipose tissue is known to be colorless.

The writers have been impressed for some time with the fact that there seemed to be more than a casual relation between the simultaneous presence of carotinoids and fat-soluble vitamine in

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[1] Schunck, C. A., *Proc. Roy. Soc. London*, 1904, lxxii, 165.

[2] Willstätter, R., and Escher, H. H., *Z. physiol. Chem.*, 1911–12, lxxvi, 214.

[3] Palmer, L. S., *J. Biol. Chem.*, 1915, xxiii, 261.

[4] Palmer, L. S., and Eckles, C. H., *J. Biol. Chem.*, 1914, xvii, 191; *Missouri Agric. Exp. Sta., Bull. 9, 10, 11, and 12*, 1914.

[5] Palmer, L. S., *J. Biol. Chem.*, 1916, xxvii, 27.

butter fat and in the leafy parts of green plants, and the absence of carotinoids and fat-soluble vitamine from lard. It was recognized, of course, that the presence of carotinoids in a number of vegetables oils, such as cottonseed oil, corn oil, etc., and the absence of fat-soluble vitamine from vegetable fat did not coincide with this relation. The wide variations between different species of animals as to the circulation of the plant carotinoids in the blood and their incorporation with fat deposited in the body tissues and egg yolk, or secreted in the milk fat also argued against any physiological significance of the plant carotinoids for the animal body. The question seemed necessarily to be an open one, however, until it could be established that these plant pigments are entirely dispensable to those species of animals in which they are naturally found.

The earlier work which one of us[3] did on the pigmentation of the egg yolk suggested that the fowl would no doubt lend itself readily to a study of the relation of the plant carotinoids to growth and reproduction in animals. The advantages as well as the difficulties attending the use of chickens for studies involving growth have been pointed out recently by Osborne and Mendel.[6] We did not anticipate the difficulties which Drummond[7] encountered in growing chickens in confinement. As a matter of fact, however, certain nutritional difficulties were encountered, which, together with one or two unfortunate accidents, prevented the successful carrying out of our first principal experiment, begun in March, 1917.

Our problem was primarily one of selecting a ration entirely devoid of carotinoids, particularly xanthophyll, but otherwise presumably adequate for normal growth. A careful examination of a large number of plant materials suitable for chick food showed that white corn is the only natural grain food which can be stated to be entirely free from pigment.[8] Certain succulent feeds such

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiii, 433.

[7] Drummond, J. C., *Biochem. J.*, 1916, x, 77.

[8] Certain samples of white corn examined contained minute amounts of carotinoid, but this may have been contamination. Bleached patent flour, which could formerly be obtained on the market, is also free from carotinoids as the result of the bleaching process. We used it to a certain extent in a portion of the experimental work.

as white onions and summer squash are probably free from carotinoid, but this was not found to be the case with Irish potatoes, although the latter contain very small quantities only. No systematic examination of animal products suitable for chicken food was made for the first experiment. Separator skim milk and meat scrap only were examined, with the view of using both in the ration. The presence of appreciable amounts of carotinoid in the meat scrap, presumably associated with the fat present, caused us to limit the animal food to the separator skim milk. Although the trace of fat in this product probably contained carotin it was judged that the fact that chickens make little if any use of this class of carotinoids, as shown previously by one of us,[3] would not interfere with the interpretation of the results in case we were successful in raising the chicks on the carotinoid-free ration.

EXPERIMENTAL.

Experiment 1.—This was a preliminary trial to study the adequacy of the carotinoid-free ration for growth. Eleven half grown, White Leghorn chickens, weighing 700 to 750 gm. (1.5 pounds), were put on a ration consisting of a mash of white cornmeal, white corn bran, and bleached flour, with white corn for scratch feed and sour skim milk and bone meal *ad libitum*. Previous to the experimental feeding the birds had been running on range in addition to receiving the regular ration of the University flock. The shanks, beaks, ear lobes, etc., of the birds were accordingly highly pigmented with xanthophyll. The birds were confined to a small brooder house, about 8 by 10 feet, on a board floor, and were given the range of a small dirt yard, the vegetation upon which had been killed by a previous application of brine. The experiment was begun in October, 1916, and was continued until March 15, 1917, when most of the females had begun to lay. By this time various causes had reduced the flock to five birds, four of which were hens. All had shown good growth during the 4 to 5 months, in most cases having practically doubled their weight.

At the time the first egg was laid on February 22, 1917, all the birds had been decidedly affected by the carotinoid-free ration with respect to the yellow skin coloring visible in shanks, beak,

ear lobes, etc. These parts which previously had been highly colored were now only faintly tinted. We have since made a careful study of the causes underlying the changes in the visible body pigmentation of fowls of the White Leghorn breed, and especially the cause of the relation of these changes to fecundity which has been studied recently from a practical point of view by Blakeslee and Warner and others.[9] The report of these studies is given in the succeeding paper.

In addition to securing preliminary data regarding the growth of chickens on the carotinoid-free ration it was desired to continue the preliminary experiment until the birds began to exhibit fecundity. We hoped thereby to observe the relation of the ration to this function and also the effect of the long continued

TABLE I.

Color of Egg Yolks on Carotinoid-Free Ration.

Date of egg.	Bird No.	Units of yellow.	Units of red.
1917			
Feb. 22	X	3.0	1.2
" 25	1885	3.0	1.2
" 27	1886	2.8	1.5
March 5	1876	2.7	1.0
" 7	1876	2.7	1.0
" 10	1876	2.7	1.1

feeding of a carotinoid-free ration on the color of the egg yolks. Three of the remaining hens had laid eggs by March 10, 1917, after which the experiment was discontinued.

The color of the yolks of the eggs was observed by spreading the raw yolk upon a white porcelain crucible cover and matching the tint with the color glasses of the Lovibond tintometer which was inclined from the egg yolk at an angle of 105°. The results are shown in Table I.

It is observed that the yolks were not absolutely colorless. They were, however, very pale, as will be readily recognized by

[9] Blakeslee, A. F., and Warner, D. E., *Science*, 1915, xli, 432; *Am. Nat.*, 1915, xlix, 360. Harris, J. A., Blakeslee, A. F., and Warner, D. E., *Genetics*, 1917, ii, 36. Blakeslee, A. F., Harris, J. A., Warner, D. E., and Kirkpatrick, W. F., *Storrs Agric. Exp. Sta., Bull. 92*, 1917.

anyone familiar with the color glasses of the Lovibond tintometer. Moreover, the color averaged lower than that secured on a ration very low in carotinoids which was reported by one of us in a previous paper.[3] The average color of the raw yolks observed in that experiment on the "non-pigmented" ration was 3.5 units of yellow and 1.2 units of red.

The conclusion was drawn at this time that the failure of the yolks to be colorless was the result of a storage of xanthophyll in the body of the fowls previous to being placed on the carotinoid-free ration. The actual cause of most of the color will be discussed below in connection with Experiment 3.

Experiment 2.—In this experiment, begun in March, 1917, a flock of 75 newly hatched chicks, mostly White Leghorns, were put on the carotinoid-free ration of white corn, bleached flour, and separator skim milk, with bone meal *ad libitum*, immediately after hatching. They were kept in a brooder pen on a board floor with a straw covering for the first few weeks and were later transferred to the brooder house and bare yard used in the preliminary experiment.

The chicks exhibited normal growth for the first few weeks. The bright yellow shanks and beaks which the chicks had on hatching rapidly disappeared until they showed no visible pigmentation in these parts except the natural pink color of living muscular tissue. Marked decline in growth and condition became evident soon after they were transferred to the brooder house. Death reduced the flock at least one-half within a few weeks. The bare dirt yard upon which they ran was spaded up, with the hope that the scratching in the loose dirt would improve the condition of the birds. This action proved to be a fatal one. Although the birds showed great activity in the loose dirt, devouring numbers of worms and bugs which were spaded up, they also ate considerable quantities of dirt and the flock was reduced to about a dozen birds, the others dying suddenly with marked evidence of salt poisoning from the brine-treated soil. One bird only, a hen, of the flock was raised to maturity, and she was lost in November, as the result of an attack of roup, before she had begun to exhibit fecundity.

Experiment 3.—It was determined to repeat Experiment 2 during the season of 1918–19. Just before the experiment was

begun a paper appeared by Osborne and Mendel[10] on the growth of chickens in confinement in which these authors stated that they had been successful in raising a few chicks from the time of hatching on artificial diets devoid of *plant* carotinoids. They noted the absence of pigmentation from the shanks and ear lobes of their birds. Inasmuch, however, as the rations were not absolutely devoid of carotin and xanthophyll, due to the liberal amount of butter fat incorporated in the ration for the purpose of supplying fat-soluble vitamine, their experiment cannot be judged to have answered the question of the possible relation of carotinoids to growth and reproduction.

Osborne and Mendel attributed their success partly to the use of blotting paper or paper pulp as a source of roughage, which their birds ate with avidity and which greatly improved the consistency of the rations which they employed. This suggestion seemed to be a happy one and we were very glad to adopt it in our 1918–19 experiment, inasmuch as our 1917 experiment failed to provide any roughage because of the impossibility of securing a natural roughage free from carotinoids. For the purposes of our study paper pulp would not only supply the roughage which the ration lacked but could be used at the same time, without introducing carotinoids into the ration.

It was felt, however, that the nutritional disturbances encountered in the previous experiment were probably due largely to the failure of the ration to supply the quantity of vitamines essential for normal growth and well-being. We believed that this was due largely to the fact that chicks do not ordinarily consume sufficient milk, in the liquid form, to provide the amount of vitamines necessary for growth. For example, it was observed that the birds would pick out the solid particles in the curdled skim milk and leave the whey, in which the bulk of the water-soluble vitamines naturally would be found. With regard to an adequate supply of fat-soluble vitamines, the ration used was judged to be somewhat deficient, but not dangerously so. It was desired to avoid the use of milk powder, if possible, because it would probably involve the additional labor of baking the food containing it. This would be necessary inasmuch as young chicks

[10] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiii, 433.

do not readily eat very finely divided material or rations which are quite pasty in character, which would be the case if the milk powder merely was mixed with a little water.

A preliminary paper by Osborne and Mendel[11] on the use of dried pig liver as the sole source of both water- and fat-soluble vitamine, which was confirmed by a later paper by the same authors[12] seemed to offer a solution of our problem, inasmuch as one of us had previously shown that the blood serum of the pig is devoid of carotinoids. A careful examination of pork liver showed no pigments to be present which were distinctly characteristic of carotin or xanthophyll, although considerable difficulty was encountered in handling the material, due to the presence of bile and liver pigments. The success which we attained by modifying the carotinoid-free ration to include pork liver is reported below.

60 newly hatched White Leghorn chicks were immediately placed on the carotinoid-free ration about April 15, 1918. This ration consisted of white corn for scratch feed, a mash of white corn-meal, and white corn bran with skim milk and bone meal and paper pulp *ad libitum*. Rice flour was used in the mash for a brief period but was discontinued following the discovery that it contained traces of carotinoids. The chicks were placed in a pen on a board floor covered with dirt and had the run of a small dirt yard, absolutely free from vegetation. The chicks soon learned to eat the paper which was frequently supplied merely by throwing newspaper into the pen.

Influence of Carotinoid-Free Ration on Growth.

The birds exhibited fair growth for the first 6 weeks when they began to decline, as in the previous experiment. A few of the flock died. At this stage pork liver was added to the ration. The raw liver was merely cut up fine and thrown into the pen. This was done every other day, no attempt being made to govern closely the quantity of liver which was fed. All the birds immediately exhibited a marked improvement in growth and condition. By July 15, when the chicks were 3 months old, nearly

[11] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309.
[12] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 17.

all the 50 remaining members of the flock were judged to be normal in size for their age. During the summer months the birds were occasionally given a little succulent feed in the form of white Spanish onions or white summer squash.

By September 1, 1918, the birds weighed on the average 1 kilo each. They appeared to be normal and vigorous in every way with the exception of the complete absence of pigmentation in the shanks, beaks, ear lobes, eye ring, and all other visible skin parts. The oil which could be squeezed out of the oil sac on the base of the tail was also entirely colorless.

Apparently the birds were absolutely free from carotinoids. A careful examination of the carcasses of several of them showed, however, that there was probably a trace of pigment in the blood serum and adipose tissue. A reexamination of the constituent parts of the ration indicated that this probably came from traces of carotinoids in the newspaper which was used for roughage and in the rice flour which was incorporated in the ration for a short time.

In spite of the fact that the rations used can be said to have contained an irreducible minimum of carotinoids only, we feel entirely justified in concluding from this experiment that fowls can be raised from birth to maturity on rations devoid or containing mere traces of carotinoids provided the ration contains an adequate supply of growth-promoting vitamines. This requirement was met in our carotinoid-free ration by the use of fresh pork liver, which contains no carotinoids.

The experiment also seems to point conclusively to the fact that the association of carotinoids and fat-soluble vitamine in certain plant and animal materials such as green leaves and butter fat, is probably fortuitous. Unfortunately also no significance can be attached to this relation as an aid in the establishment of the identity of the fat-soluble vitamine, which is so greatly to be desired.

Influence of Carotinoid-Free Rations on Fecundity.

The females in the pigmentless flock began to exhibit fecundity just 6 months after hatching. Two of the pullets dropped eggs on October 11, 1918. The flock contained about 30 females,

all of which have apparently exhibited normal fecundity on the carotinoid-free ration upon which they have been fed since hatching. The total egg record of seventeen hens of this flock from October 11, 1918, to May 22, 1919, a period of 223 days, has been 893 eggs, an average of 52.5 eggs per hen. Several of the birds have considerably higher records. For example, Hen 2922 which began laying November 8, has a record of 88 eggs up to May 22; and Hen 2950, which began laying November 14th, has a record of 82 eggs up to May 22. Hen 2942 whose egg record was kept for a shorter period on a special carotinoid-free ration, which will be discussed below, laid 44 eggs in a period of 59 days. These records are as good as could be desired under normal conditions.

Our observations regarding the fecundity of the birds on carotinoid-free rations unquestionably justify the conclusion that the absence of carotinoids from the ration from birth to maturity has no deleterious effects on fecundity; and also that this function is not interfered with by failure to provide carotinoids during the period of egg laying activity.

Influence of Carotinoid-Free Rations on Color of Egg Yolk.

Considerable interest naturally was attached to the character of pigmentation of the egg yolk from hens raised on rations which had provided no yellow plant pigments for the body stores from the time of hatching.

When one examines the hard boiled yolk of the eggs the entire yolk has the appearance of the so-called white yolk of the hen egg, in which no pigment of any kind is normally present. The raw yolk of the carotinoid-free egg has, however, a pale yellowish color. In the terms of the Lovibond tintometer color units we have found the average color of a large number of raw yolks of these eggs to be 2.0 units of yellow, 0.3 unit of red. It was found, however, that the comparative examination of the color of a large number of raw egg yolks was made more conveniently with the Bradley color top. This simple toy has been used successfully by Blakeslee, Harris, Warner, and Kirkpatrick[9] for matching the color of the ear lobes of White Leghorn hens in their study of the pigmentation of the ear lobes as a criterion for the

selection of laying hens. Although the colors used on the paper disks of the top are by no means pure, very useful comparative results can be secured with them. In recording the color of the raw yolks the mixed material was spread out in a 5 cm. porcelain crucible cover and the color matched by the spinning top with the top and yolk resting on a white background using good daylight but not direct sunlight for illumination. Some of the

TABLE II.

Color of Raw Yolks on Carotinoid-Free Ration (1918–19 Experiment).

Date of egg.	Bird No.	Color of raw yolk.			
		Yellow.	White.	Green.	Orange.
1918		*per cent*	*per cent*	*per cent*	*per cent*
Oct. 28	2944	65	35	0	0
Nov. 16	2945	65	28	7	0
Dec. 17	2945	60	35	5	0
Nov. 1	2936	65	28	7	0
Dec. 18	2936	65	28	7	0
Oct. 28	2914	50	40	10	0
Nov. 14	2950	65	25	10	0
Dec. 18	2950	65	30	5	0
Nov. 29	3136	65	28	7	0
" 8	2922	60	30	10	0
" 28	2922	65	30	5	0
Dec. 17	2922	60	30	5	0
1919					
March 4*	2922	55	40	5	0
" 13*	2942	60	35	5	0
April 22*	2942	65	30	5	0
Feb. 25*	2947	65	35	0	0
April 20*	2947	60	37	3	0
May 22	Normal yolk.	60	0	0	40

* Special feed of skim milk powder and corn-starch.

many observations made in this manner are recorded in Table II. The color of a normally pigmented yolk is given also, for the sake of comparison.

It did not seem likely that the small amount of residual pigment in the yolks of these eggs was being derived from the feed. Nevertheless three of the best layers were selected for the study of a special ration designed to be absolutely free from carotinoids.

This ration was constructed from skim milk powder, corn-starch, filter paper, and bleached butter fat after calculating the protein, carbohydrates, fat, crude fiber, and ash content of a ration consisting of 10 pounds of corn, 5 pounds of barley, 3 pounds each of wheat bran and ground oats, and 3 gallons of milk, a ration regarded as satisfactory for 100 laying hens. The skim milk powder contained 0.5 per cent fat. Enough of the ingredients to supply three hens for 1 week was mixed together at one time without the bone meal, which was fed separately *ad libitum*, sufficient water added to make a dough, and the material baked in thin layers for 20 to 30 minutes at 120°C. The cakes were dried, ground to small pieces in a food chopper, and fed as the sole ration to the laying hens.

After continuing this ration for a month without reducing the color of the yolks, the ration was changed so that it contained no filter paper or fat, and the bone meal was changed to ground limestone. The hens continued to lay heavily even without any fat other than the 0.5 per cent fat in the skim milk powder. No reduction in the color of the yolks was effected as the result of this modified ration. Some of the color top readings of the egg yolks from Hens 2922, 2942, and 2947 on these special rations are shown in Table II. It is worthy of note also, that it was while on this special ration that Hen 2942 laid 44 eggs in 59 days. The birds were kept in separate pens, about 3 by 8 feet, on a sand floor, throughout the special feeding.

Nature of Residual Egg Yolk Pigment.

It seemed certain, at least from a physiological standpoint, that the residual pigment in the yolks was not derived from plant carotinoids. This was repeatedly confirmed by the observation that the acetone extract of the egg yolks leaves a yellow-colored oil which fails to reduce $FeCl_3$ crystals to the green ferrous salt like the extracts of yolks which are known to contain xanthophyll.

Barbieri[13] has described a water-soluble pigment obtained from eggs, which he believes to be the cause of the natural yellow color of the yolk. According to Barbieri this pigment, which is

[13] Barbieri, N. A., *Compt. rend. Acad.*, 1912, cliv, 1726.

called ovochromine, is, however, insoluble in alcohol, ether, chloroform, acetone, etc. It was attempted at first to associate the residual, non-carotinoid pigment of the yolks with Barbieri's ovochromine, but we were forced to abandon this idea because of the ease with which acetone readily extracts the pigment from the raw yolk.

An attempt was also made to identify the residual pigment with bilirubin because of the fact that it appears to be only partly extracted with the fat of the yolk by ether. The pigment failed, however, to give Gmelin's test for bilirubin or to be extracted from its chloroform solution by dilute alkali.

The residue from the hot acetone extract of the carotinoid-free yolks could be separated into a major golden yellow fraction on shaking with ether, and a minor greenish yellow fraction soluble in water. This latter solution gave a characteristic bluish green ring with fuming nitric acid and was precipitated from its solution by mercuric chloride. Lime-water, however, failed to precipitate the pigment from alkaline solutions which is a characteristic property of the alkaline salts of bilirubin.

We regard it as unfortunate that it has so far been impossible to identify the residual yellow pigment of the carotinoid-free eggs with any of the well known animal coloring matters. There is no question, however, of the non-identity of the pigment with the carotinoids, notwithstanding the solubility of the pigment in some of the fat solvents. The two outstanding differences between the residual pigment and the carotinoids are, (1) the failure to give the characteristic reduction test with $FeCl_3$, and (2) the fact that it is possible to recover only a small fraction of the pigment with ether after saponification of the egg oil which is extracted from the yolks with the pigment by acetone.

Relation of Carotinoid-Free Rations to Fertility of the Eggs.

The cockerels which formed a part of the carotinoid-free flock were never separated from the hens, so that an opportunity was offered to study the fertility of the carotinoid-free eggs from the standpoint of both the hen and the cockerels which fertilized them. 74 of the eggs, 28 of which were laid by the three hens on the special ration of skim milk powder and corn-starch, were

placed in the incubator on May 9, 1919. 51 eggs proved to be fertile and 35 livable chicks were hatched on May 30 and 31, 1919. A second lot of 18 eggs, all from the hens fed the special ration of corn-starch and skim milk powder, were placed in the incubator on May 24. 17 eggs proved to be fertile and 6 livable chicks were hatched on June 14. The results indicate a somewhat lower hatchability for the eggs from the hens on the special carotinoid-free ration of corn-starch and skim milk powder than from those on the ration of white corn, pork liver, and skim milk.

All the chicks which hatched in both settings were vigorous, normal individuals in every way except for the complete absence of pigmentation in the shanks, beaks, and other skin parts.

The young chicks have been handled so far in every way similar to the first generation, except for the fact that they were fed for the first few days the yolks of eggs laid by the hens on the carotinoid-free ration. Later they were changed to white cornmeal and skim milk with bone meal and grit. It is planned to begin the feeding of the paper pulp and pork liver as soon as the chicks are old enough to handle these materials.

At the time of writing the chicks are several weeks old and are growing normally, but do not appear to be in the best of condition. A number have died. It is planned to continue the study, if possible, through the second generation, and probably also through the third generation.

Fig. 1, Plate 2, shows Hens 2942 and 2947 and one of the full grown cocks at the age of 17 months. A portion of the flock of the two hatchings of baby chicks of the second generation at the ages of 10 and 24 days, respectively, is shown in Fig. 2.

SUMMARY.

1. White Leghorn chicks have been raised from hatching to maturity on rations containing the merest traces, if not entirely devoid, of carotinoids. The full grown hens have shown normal fecundity, and no abnormalities with respect to the fertility of the carotinoid-free eggs have developed. A second generation of chicks, free from carotinoids at hatching, has been started with every evidence of being normal except for the absence of the natural yellow pigmentation of the skin.

2. It is concluded that the natural yellow pigment of fowls which is derived from the xanthophyll of the food bears no important relation to growth or to the functions of fecundity and reproduction, at least for one generation.

3. When the yolks of hen eggs are devoid of their natural xanthophyll pigmentation, as the result of the absence of carotinoids from the food, a small residual pigmentation of the raw egg yolks is observed. This pigment is readily extracted by acetone but attempts to identify it with bilirubin or other known yellow animal coloring matters have so far been unsuccessful.

We wish to acknowledge the assistance in Experiment 3 of E. L. Dakan, now in charge of Poultry Husbandry at the South Dakota Agricultural Experiment Station, to whose careful supervision of the young chicks we attribute a large share of our success in raising them to maturity.

EXPLANATION OF PLATE 2.

FIG. 1. Full grown cock and Hens 2942 and 2947, raised from hatching on rations devoid of carotin and xanthophyll.

FIG. 2. Chicks hatched from eggs free from carotinoids, at ages of 10 and 24 days, respectively.

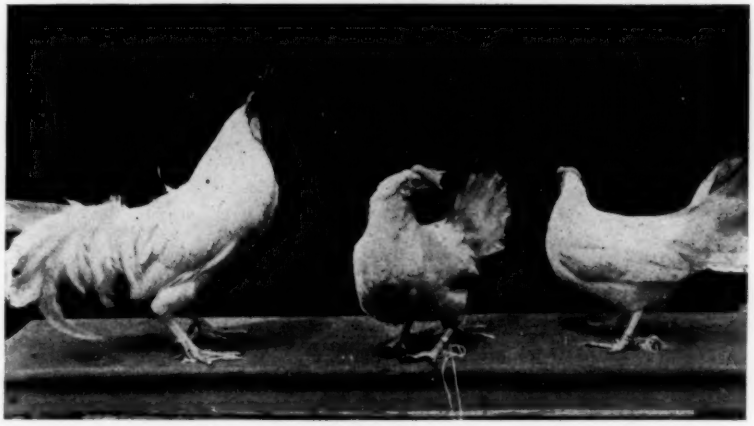

FIG. 1.

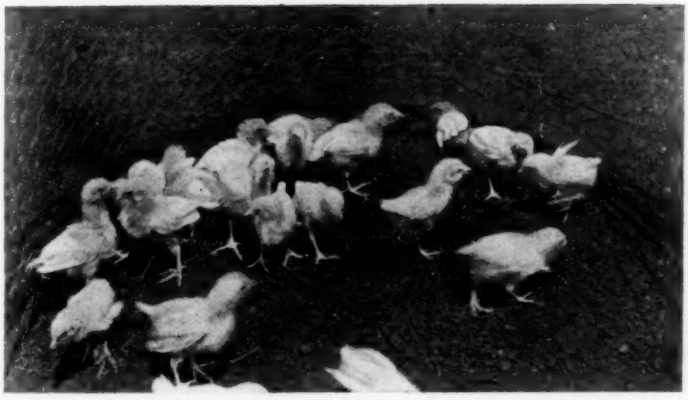

FIG. 2.

(Palmer and Kempster: Relation of carotinoids to growth.)

THE PHYSIOLOGICAL RELATION BETWEEN FECUNDITY AND THE NATURAL YELLOW PIGMENTATION OF CERTAIN BREEDS OF FOWLS.*

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Plates 3 and 4.

(Received for publication, July 9, 1919.)

Practical poultry men have recognized for several years that a close relation exists between the amount of yellow pigment to be seen in the shanks, ear lobes, beaks, etc., of hens of certain breeds of poultry, such as the Leghorns, Plymouth Rocks, Wyandottes, and Rhode Island Reds, and their previous laying activity.

Rice,[1] Kent,[2] and others have believed that heavy laying causes the leg color of these breeds of fowls to fade and have made use of this belief in selecting heavy from light laying hens at the close of the laying season.

Blakeslee and Warner[3] and the same authors with Harris and Kirkpatrick[4] have made extensive biometric analyses of data collected by them, to ascertain the correlation existing between the color of the ear lobes, shanks, beak, etc., and previous egg laying activity. The data were collected at the Storrs Agricultural Experiment Station egg laying competitions during a period of several years. These data show that in hens which naturally carry yellow pigmentation in their ear lobes, shanks, beak, etc. a positive correlation exists between pale shanks, ear lobes, beak, etc. and a recent more or less heavy egg production. The data indicate, also, that practical use may be made of this correlation for the selection

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[1] Rice, J. E., *N. Y. State Dept. Agric. Circular 54*, 1912.

[2] Kent, O. B., *J. Am. Assn. Instructors and Investigators of Poultry Husbandry*, 1916, ii, 63; *Am. Poultry World*, 1915, ix, 499, 534.

[3] Blakeslee, A. F., and Warner, D. E., *Science*, 1915, xli, 432; *Am. Nat.*, 1915, xlix, 360. Warner, D. E., *Field*, 1916, xxvi, 24.

[4] Blakeslee, A. F., Harris, J. A., Warner, D. E., and Kirkpatrick, W. F., *Storrs Agric. Exp. Sta., Bull. 92*, 1917, 95.

of heavy laying from light laying hens. The results show, however, that this can be done *only* when the observations regarding pigmentation are made *immediately* after the close of the laying season.

We have been interested in this interesting phenomenon from a physiological as well as a practical standpoint. The hypothesis which has been advanced[5] and which has been adopted by Blakeslee and Warner[3] in explanation of the relationship which has been observed between fecundity and pigmentation is that the growth of the egg abstracts the pigments from the body tissues with the resulting negative correlation between egg production and quantity of yellow pigment present in the ear lobes, beak, shanks, etc. In support of this hypothesis these authors[4] have cited the histological work of Barrows[6] who states that the yellow shank color of fowls is due to yellow fat deposits in the Malphigian layer of the epidermis, and the investigation of one of us[7] which established the physiological relation between the xanthophyll of plants and the lipochrome of the egg yolk, blood serum, and body fat of the hen. The ease with which it has been found possible to transmit fat-soluble dyes, such as Sudan III, to the egg yolk and body fat of fowls by feeding, as shown by Riddle,[8] Gage and Gage,[9] Rogers,[10] and Mendel and Daniels,[11] has also been cited in favor of the hypothesis mentioned. In addition Warner and Edmond,[12] and Riddle and Harris[13] have studied the percentage of fat in the blood of laying and non-laying birds, with the result that Warner and Edmond believe that the high percentage of fat in the blood of laying hens, as compared with those which are not laying, supports the hypothesis that the tissue fat is being transferred to the egg yolk during heavy laying with a consequent subtraction of pigment. The low percentage of fat in the blood of non-laying birds is believed to show, on the other hand, that birds which are not laying are storing fat in the body cells, and in consequence are increasing the yellow color which is naturally found in the body tissues. In this connection, Blakeslee, Harris, Warner, and Kirkpatrick[4] differentiate between two groups of domestic fowls, namely the American breeds and Leghorns, which are naturally yellow in the visible skin parts, and the English Orpington breed in which, "yellow is constantly absent from legs, beak, *and body fat.*"[14] The statement that the

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[5] Anonymous, *Maine Agric. Exp. Sta. Press Letter 144*, 1914.

[6] Barrows, H. R., *Maine Agric. Exp. Sta. Bull. 232*, 1914, 237.

[7] Palmer, L. S., *J. Biol. Chem.*, 1915, xxiii, 261.

[8] Riddle, O., *J. Exp. Zool.*, 1910, viii, 163.

[9] Gage, S. H., and Gage, S. P., *Science*, 1908, xxviii, 494.

[10] Rogers, C. A., *Proc. Internat. Assn. Instructors and Investigators in Poultry Husbandry*, 1912, i, 77.

[11] Mendel, L. B., and Daniels, A. L., *J. Biol. Chem.*, 1912–13, xiii, 71.

[12] Warner, D. E., and Edmond, H. D., *J. Biol. Chem.*, 1917, xxxi, 281.

[13] Riddle, O., and Harris, J. A., *J. Biol. Chem.*, 1918, xxxiv, 161.

[14] The italics are ours.

body fat, as well as the shanks, of birds of the Orpington breed is colorless is, of course, totally incorrect, their body fat being as yellow as that of the yellow shanked breeds.

The success which we attained in raising a flock of White Leghorn fowls to maturity with the complete absence of carotinoids from the body, as the result of excluding these pigments from the food, as reported in the preceding paper,[15] seemed to offer an opportunity to ascertain the true physiological relation between fecundity and the fading of the xanthophyll pigmentation of the skin. We have from the first doubted the accuracy of the hypothesis that laying abstracts the xanthophyll from the shanks, beak, ear-lobes, etc., and excretes in it the egg yolk, although we accepted the statement of Barrows that the pigment in the visible skin parts is deposited as yellow fat. As the result of our study of this phenomenon, however, we have been led to adopt an entirely different hypothesis. We now believe that the fading of the yellow pigmentation in the visible skin parts as the result of fecundity in breeds such as the Leghorns is *not* due to a subtraction of pigmented fat from these parts and its subsequent incorporation in the egg yolk. On the other hand we believe that the normal appearance of xanthophyll in the beaks, shanks, ear lobes, etc. of the yellow-colored breeds is the result of a natural excretion of pigment. Fecundity merely deflects this path of excretion to the egg yolk thereby removing the source of the pigment for the skin. The result is that the pigment found in the skin at the onset of fecundity is gradually excreted towards the epidermis where it either becomes oxidized (decolorized) or wears away as the result of the normal structural changes of the epidermis.

We desire to present in this paper the evidence which we have accumulated in support of this hypothesis.

EXPERIMENTAL.

Effect of Removing the Supply of Pigment.

Blakeslee, Harris, Warner, and Kirkpatrick[4] have stated that, "Lack of green food or other substances necessary for pigment formation as well as other environmental factors may cause a

[15] Palmer, L. S., and Kempster, H. A., *J. Biol. Chem.*, 1919, xxxix, 299.

fading of the yellow [from ear lobes, shanks, etc.] entirely independent of any laying activity." These authors have failed to appreciate, however, that this fact is of fundamental importance in explaining the fading of the skin pigment during fecundity. We proposed to demonstrate experimentally that the removal of the supply of xanthophyll from the food causes a gradual reduction in the amount of xanthophyll in the ear lobes, beak, and shanks, independent of fecundity; and furthermore that this reduction is the result of a movement of pigment to the outer part of the epidermis, with the resulting wearing away and oxidation or bleaching of the xanthophyll.

For this purpose two full grown cockerels of the carotinoid-free flock were confined to small exhibition cages and fattened for a period of 16 days on a heavy ration of white corn and skim milk. The floor of the cages was kept covered with clean sand. Bird 482 gained in weight from 1.30 to 1.50 kilos, and Bird 480 from 1.25 to 1.50 kilos. The white corn was then changed to yellow corn.

On the 5th day of yellow corn feeding the shanks and beak had begun to show a yellow color. On the following day it was distinctly visible in the ear lobes, eye rings, and also in the comb when the blood was pressed away from a small portion. Bird 480 was killed on the 8th day of xanthophyll feeding and an abundance of xanthophyll found in the blood serum, and in the Malphigian layer of the epidermis of the beak, ear lobes, comb, and shank. The adipose tissue was also somewhat colored with xanthophyll, a 1 inch layer of the rendered, melted fat showing a color of 4.2 units of yellow and 0.8 unit of red, when viewed through the Lovibond tintometer.

The yellow corn feeding was continued with Bird 482 for 42 days. By this time not only were the shanks, beak, ear lobes, and vent highly colored but the feathers, also, had taken on a distinctly creamy appearance, and the comb had changed from a blood-red to an orange-red color. In connection with the beak coloring the observation was made that its pigmentation began at the base and only slowly extended out towards the tips, several weeks elapsing before the entire beak was colored.

Bird 482 and four other equally highly colored White Leghorn cockerels from the University flock were then confined to sep-

arate small exhibition cages and put on a ration of white corn and skim milk. It was planned to kill these birds at intervals during the gradual fading of the visible pigmentation which was anticipated, and determine the quantity of xanthophyll in the blood serum, and particularly to make a comparative histological study of the relative distribution of the xanthophyll in the several parts of the skin, particularly of the shank.

At the beginning of the white corn feeding the ear lobes of the birds averaged 60 per cent yellow, using the Bradley color top for measuring the color. After 60 days of white corn feeding it was no longer possible to detect any color in the ear lobes, by this method. The combs were also free from pigment and the base of the beaks was distinctly lighter in color than the tip. The shanks, also, had begun to show a noticeable fading and the feathers, which had been smoothly creamy, were now mixed white and yellowish and almost colorless at the base of the quills. Cock 2656 was killed at this time. The blood serum was found to be free from xanthophyll. The adipose tissue, however, was distinctly yellow.

90 days after the change to carotinoid-free ration the beak, ear lobes, comb skin, eye ring, and vent of the birds were entirely colorless and a marked decolorization of the shank skin had also taken place. Bird 2692 was killed at this time. The blood serum was not examined. The adipose tissue, however, was yellow, but not highly colored.

Bird A of the group died 135 days after the change to carotinoid-free ration. Frozen sections of shank skin only were made of this bird. These will be discussed below.

The two remaining birds of the flock were kept on the white corn and skim milk until their shanks had completely bleached out. One of these was Bird 482, which was raised on carotinoid-free ration, heavily pigmented with xanthophyll by feeding yellow corn, and then changed back to a carotinoid-free ration. After 200 days no pigment remained in the shanks except that deposited under the thick, horny scales on the sides of the legs. If these birds had had thinner shank skins like the females of this breed, their shanks would no doubt have been entirely colorless also by this time. Fig. 1, Plate 3, shows Bird 482, after 275 days of confinement in a small exhibition cage.

We attach great importance not only to the gradual disappearance of xanthophyll from the beak, ear lobes, comb, vent, and shanks when the supply of xanthophyll is cut off in the food, but particularly to the microscopic appearance of the frozen vertical sections of shank skin. A place near the bottom of the foot was selected for these sections because of its deep pigmentation in highly colored birds, and also because the epidermis and corium layers at this point do not have such a strong tendency to separate during staining, as sections made from the side of the shank where the epidermis is very horny.

Sections were made, after killing, of the foot skin of a highly pigmented bird and of Birds 2656, 2692, and A during the various stages of decolorization. Sections were also made from the feet of Bird 482 after they had completely lost their color. This was done, however, by cutting a small vertical piece of skin and flesh from the bottom of the foot without killing the bird.

As Barrows[6] has previously shown, we found the xanthophyll to be located in the shank skin chiefly in the rete of Malphigi with some pigment in the upper part of the epidermis and some diffusion of pigment through the corium. Especially instructive were these sections after staining with Nile blue. The Nile blue which we used was found to be dichromatic with respect to fat and xanthophyll, the fat staining red and the xanthophyll deep blue. Fig. 2, Plate 4, represents the highly pigmented shank skin stained with Nile blue. The xanthophyll is seen to be deposited in the outer part of the epidermis and especially in the cells of the rete of Malphigi largely as dark blue granular masses. These granular masses are also seen lining the blood capillaries in the lower corium layer. The fat in the subcutaneous tissue stands out prominently as scarlet-colored droplets. Some sections were made which showed a reddish tinge in the epidermis indicating the presence of some fat. It is possible that here the fat is dissolved in xanthophyll rather than xanthophyll dissolved in fat which is the case in adipose tissue of normal chickens.

The pigment in the epidermis was identified as carotinoid by its ease of oxidation and decolorization with $FeCl_3$[16] and hydrogen peroxide.

[16] Palmer, L. S., and Thrun, W. E., *J. Ind. and Eng. Chem.*, 1916, viii, 614.

Further proof of the fact that xanthophyll in the shank skin is not dissolved in fat was secured by observing the character of stain which the Nile blue gives to fat which had been deeply colored with carotinoids. Butter fat or lard highly colored from contact with dried carrot powder and the fat in deeply colored egg yolk took a red stain from the Nile blue showing that carotinoids dissolved in fat do not influence the normal stain which the fat takes from this dye.

Especially significant was the behavior of Sudan III when fed for 8 days to one of our pigment-free cockerels. No trace of the dye was found in the ear lobes, beak, comb, vent, or shanks, although the adipose tissue and bone marrow were found to have a deep pink color after the bird was killed.

Blakeslee[17] reports a similar result after 10 days of feeding Sudan III to two laying hens,[18] but explains the failure of the dye to appear in the ear lobes, etc., on the ground that the xanthophyll-free epidermis lacks stainable fat. Gage and Gage,[9] Salant and Bengis,[19] Mendel and Daniels,[11] and others who have fed Sudan III have failed to find the dye deposited in any parts of the body except the adipose tissue. It is true that xanthophyll when present in the epidermis will take a stain characteristic of fat when frozen sections of the skin are treated with Sudan III and scarlet-red, but it is not possible to interpret this as indicative of a solution of xanthophyll in fat inasmuch as Nile blue fails to give a characteristic fat stain to the pigment, and the fat-soluble dyes fail to be deposited in the visible skin parts.

As the xanthophyll gradually faded from the shank skin of the cockerels on the carotinoid-free ration the microscopic observations of the stained frozen sections showed first a disappearance of pigment from the corium, then a disappearance of pigment from the outer layer of epidermis which gradually extended into the rete of Malphigi, the last pigment to disappear being the xanthophyll at the base of the Malphigian layer. Fig. 3, Plate 4, shows the appearance of a section of the decolorized shank skin

[17] Blakeslee, A. F., *Storrs Agric. Exp. Sta., Bull. 92*, 1917, 152.

[18] We have also fed Sudan III to a laying hen with the result that both the adipose tissue and egg yolk were highly colored with the dye, but the visible skin parts were not affected.

[19] Salant, W., and Bengis, R., *J. Biol. Chem.*, 1916, xxvii, 403.

of Bird 482, after staining with Nile blue. Only a pale blue diffused coloring of nuclei in the Malphigian layer is seen, with red-colored fat droplets in the subcutaneous tissue.

We interpret these observations to mean that when the supply of xanthophyll is cut off by reason of its removal from the food any pigment present in the corium layer of the skin of the shank, ear lobes, etc., is normally deposited in the rete of Malphigi. At the same time the xanthophyll deposits in the outer layer of the epidermis either wear off by reason of the normal replacement of the outer cells from those lower down, or is oxidized because of the closer contact with air. Possibly both factors are of importance in the disappearance of the color from the outer part of the epidermis. The xanthophyll deposits in the rete of Malphigi gradually become a part of the outer layers of epidermis and are lost also, until the skin finally becomes free from visible yellow pigment.

That the pigment in the adipose tissue of the fading birds is not related to the disappearance of pigment from the skin is shown by the fact that the adipose tissue still retains its normal color after the visible skin parts have lost their pigment completely. Cock 2699, one of the yellow-colored birds taken from the University flock and kept on carotinoid-free ration, together with Cock 482, until the visible skin parts had faded, was killed after 220 days on this ration. The blood plasma was free from pigment, but the adipose tissue was yellow. The rendered, melted fat showed a color of 28.0 units yellow, 1.0 unit red in a 1 inch layer in the Lovibond tintometer.

Effect of Feeding Carotinoids during Fecundity.

If fecundicity deflects the path of excretion of xanthophyll from the skin to the ovaries and thereby removes the supply of pigment for the shanks, etc., it is to be expected that no restoration of xanthophyll in these parts can be effected by feeding pigment so long as fecundity continues. According to this hypothesis a continuous fecundity only is required for the fading of all the colored skin parts of the hen, whether this results in the laying of a large or a small number of eggs.

Definite proof of the correctness of this hypothesis was furnished

by the effect on the body and skin color of our laying carotinoid-free hens, through the feeding of rations rich in xanthophyll. A number of the laying hens of the flock which was fed rations devoid of carotinoids from hatching were confined to separate cages and placed on rations containing more or less xanthophyll. Interest is attached particularly to the results secured from the hens which were fed an abundance of green feed and yellow corn, respectively, both of which are rich in xanthophyll. The egg records, color of egg yolks, color of body fat, and amount of pigment in the blood plasma of these hens after 28 days of feeding the above rations are given in Table I. Similar data are shown

TABLE I.

Effect of Feeding Xanthophyll to Xanthophyll-Free Laying Hens.

Bird No.	Character of feed.	Number of eggs laid.	Color* of egg yolks.		Color† of blood plasma.		Color‡ of body fat.	
			Yellow.	Red.	Yellow.	Red.	Yellow.	Red.
2950	Green feed.	15	47.0	4.5	10.0	1.2	3.5	0.2
3152	Yellow corn.	10	33.0	4.2	7.0	0.8	2.2	0.2
2906	Red "	14	5.5	1.5	—	—	1.2	0.2

* The color of the egg yolks is that of the raw yolk in terms of the color units of the Lovibond tintometer set at an angle of 105° from the surface of the yolk.

† The color of the blood plasma is that of the ether extract of 5 cc. of the desiccated oxalated plasma concentrated to 12.5 cc., in 1 inch layer, in terms of the color units of the Lovibond tintometer, set at an angle of 45° from horizontal.

‡ The color of the body fat is that of a 1 inch layer of rendered, melted fat in terms of color units of the Lovibond tintometer, set at an angle of 45° from horizontal.

for the hen fed red corn containing relatively small amounts of xanthophyll.

Of special importance, however, is the fact that no pigment whatever was deposited in the ear lobes, beak, comb, vent, or shanks during this period. Each of the birds was as colorless at the end of the xanthophyll feeding as at the beginning as far as visible yellow pigmentation was concerned although the egg yolks were very highly colored. We were especially astonished to find that the adipose tissue which was abundant in each of the hens,

especially in the abdominal cavity, was also almost as colorless at the close of the xanthophyll feeding as in the case of the birds which had never received carotinoids since hatching.

We had anticipated the failure of the xanthophyll-rich rations to color the ear lobes, beak, shanks, and other visible skin parts during fecundity but were not prepared for the additional confirmation of the hypothesis that fecundity deflects the xanthophyll excretion to the egg yolk, by finding that the adipose tissue also fails to receive any appreciable amount of pigment.

The data in Table I show that the rendered, melted body fat of the birds showed a faint coloration. This was not visible when the fat had solidified, and even in the case of the hen fed green feed is to be regarded as almost negligible.

The bile of Hen 2950 on green feed was examined for the presence of xanthophyll after drying with lime water. The chloroform extract of the dry material left a pale yellow, oily residue on evaporation but it did not give a characteristic test for carotinoids with $FeCl_3$. This result indicates that the bile is not to be regarded as a path of excretion of xanthophyll during fecundity.

Shank, Beak, and Ear Lobe Color as Criterion of Fecundity.

We have collected a limited but very instructive amount of data on the question of pigmentation as a criterion of egg laying ability. Two laying White Leghorn hens were selected from the University flock in October, 1918, as heavy and light laying birds, respectively, without reference to their trap nest records for the season. Hen 2824 had very pale shanks and colorless ear lobes, beak, etc., and was judged to be a heavy layer. Hen 2754 had very yellow shanks, yellow beak, and pale yellow ear lobes, and was judged to be a light laying hen. Both hens had been on the regular flock ration, containing yellow corn, and had thus received some xanthophyll in their ration. The birds were bled to death and observations made of the color of the body fat, amount of pigment in the blood serum, and the pigmentation of the shanks, beak, ear lobe, etc. These observations are shown in Table II, together with the color of the raw egg yolks of the eggs laid just previous to killing, and the total egg production of the hens for the season.

The total egg production of the two birds shows clearly that body pigmentation was not the proper criterion by which to judge their relative merits as egg producers. That the birds were receiving equal amounts of xanthophyll in their ration is shown by the fact that there was no difference between the color of the egg yolks. Aside from the difference in shank, beak, and ear lobe coloring the outstanding difference between the birds is seen in the much greater pigmentation of the body fat of the yellow shanked bird. Both birds showed a low pigmentation of blood

TABLE II.

*Body Pigmentation in Relation to Fecundity.**

Observation.	Bird 2824.	Bird 2754.
Beak.	Colorless.	Tip colorless, base yellow.
Eye ring.	"	Faintly yellow.
Ear lobes.	"	5 per cent yellow.
Shanks.	30 per cent yellow.	60 " " " 30 " " red.
Oil sac.	Empty. Ether extract faint yellow.	Small amount deep yellow oil.
Blood serum.	1.5 yellow, 0.1 red.	0.7 yellow, 0.0 red.
Body fat.	12.0 " 0.7 "	47.0 " 1.0 "
Egg yolks.	13.0 " 2.4 "	12.0 " 2.3 "
" record for season.	134	131

* The skin coloring was measured with the Bradley color top, the color of the serum, body fat, and egg yolks with the Lovibond tintometer, the egg yolk colors being taken on the raw yolk with the tintometer set at an angle of 105° from the surface of the yolk.

serum, that of the white shanked bird actually being noticeably the greater.

The explanation of these apparent inconsistencies is readily seen when one compares the trap nest records of the two birds. These are shown in Tables III and IV. It is evident from these data that the reason Hen 2754 had yellow shanks and beak and pale yellow ear lobes at the end of October while Hen 2824 had white shanks, beak, ear lobes, etc., is not because there was a wide difference between their egg production but because Hen 2754 had lost her fecundity for a sufficiently long period during July, August, and

TABLE III.

Trap Nest Record of White Shanked Hen 2824.

Date.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	Total.
1919																																
Feb.													1				1	1		1		1		1	1		1					8
March.	1		1		1	1	1		1	1		1	1	1	1	1		1	1		1	1		1		1	1	1		1	1	22
April.			1	1				1	1			1	1	1		1	1				1	1	1		1	1	1	1	1			18
May.	1		1	1	1		1	1				1	1			1	1		1	1		1	1	1		1	1		1	1		19
June.							1					1			1			1	1		1	1		1	1		1	1	1			12
July.	1	1		1	1	1		1	1	1		1	1	1		1	1			1	1		1		1	1						18
Aug.																										1		1	1	1		4
Sept.	1	1		1	1		1	1		1				1	1		1	1	1		1	1			1	1	1		1	1		19
Oct.		1		1	1		1	1		1		1		1	1		1		1			1		1		1		*				14
Total for season																																134

* Bird killed.

TABLE IV.

Trap Nest Record of Yellow Shanked Hen 2754.

Date.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	Total.
1918-19																																
Dec.							1			1					1					1		1	1		1	1		1	1		1	11
Jan.	1		1		1		1		1	1		1				1																8
Feb.														1	1		1	1	1		1	1		1	1		1					10
March.		1	1			1		1	1		1	1	1	1		1	1	1		1	1	1	1		1	1	1	1		1	1	22
April.	1			1	1	1	1		1	1		1	1	1		1	1	1			1		1	1	1	1		1	1			20
May.		1		1		1	1		1	1	1	1	1	1		1	1	1	1				1		1		1	1		1		14
June.		1	1		1	1			1	1	1								1		1		1		1		1	1				14
July.			1	1		1	1		1		1	1		1		1	1		*													10
Aug.																													1	1		2
Sept.																																
Oct.	1		1	1		1	1		1	1		1	1	1		1	1		1	1		1	1		1	1		1	1†			20
Total for season																																131

* Broody

† Killed, egg in oviduct.

September to restore the normal path of pigment excretion to the shanks, ear lobes, etc., and had thus regained her skin pigmentation. Although her egg production had been excellent from September 28 until she was killed insufficient time had elapsed for her shanks to begin to lose their color, although the data in Table II show that the ear lobes had again become quite pale and her beak had also begun to fade. Hen 2824, on the other hand, lost her fecundity for a very short time only, if at all, during the last of July and the first part of August, so that she went into October and up to the time she was killed with the pale shanks, etc., which had resulted from the deflection of pigment from these parts during her continuous fecundity since the 13th of February. Had Hen 2754 been judged on the basis of color on July 18th, there is no doubt that she would have been judged to be a good layer also because of her pale shanks, and colorless ear lobes. The higher body fat color of Hen 2754 is also accounted for by her period of lost fecundity during the green feed season.

Blakeslee, Harris, Warner, and Kirkpatrick[4] recognize that skin pigmentation becomes less and less a criterion of egg production as the time of judging is removed from the date when the hen ceased to lay.

Our own conclusions regarding body pigmentation as a criterion of egg production is that the absence of pigment is more of an index of *continuous* fecundity than of *heavy egg laying*.

DISCUSSION.

The observations which are presented in this paper on the relation of pigmentation to fecundity in fowls show conclusively that it is not necessary for fowls to be exhibiting fecundity in order to lose completely the natural yellow color of the skin which is especially visible in the ear lobes, beak, comb, and shanks. It is necessary merely to withdraw the supply of pigment by withdrawing the carotinoids from the ration. Moreover, when one follows the gradual fading of the color of the shanks under these conditions by means of histological sections it is seen that the path of removal of pigment is outward and is not a resorption into the circulation. One finds, also, that the pigment in highly pigmented shanks is deposited in granular form with little or no fat present,

chiefly in the Malphigian layer of the epidermis, although some pigment granules are found also along the blood capillaries of the subcutaneous tissue.

It is to be supposed, therefore, that the xanthophyll in the blood serum is secreted into the intercellular spaces from the blood capillaries and finds its way into the cells of the Malphigian layer of the epidermis, for the most part with little or no fat. Where fat is formed the pigment is dissolved by it because of the ready solubility of carotinoids in neutral fat. As the skin loses its color, the pigment disappears first from the outer epidermis and from along the blood capillaries of the subcutaneous tissue. The pigment granules at the base of the Malphigian layer are the last to disappear.

Obviously these observations coincide with the fact that the pigment disappears much more rapidly from the thin skin of the ear lobes and comb than from the beak and shank which have a much heavier horny layer.

The application of these observations to a like disappearance of xanthophyll from the ear lobes, beak, shanks, etc., of laying hens naturally leads to the conclusion that this phenomenon is also based on a removal of the supply of pigment for the skin induced by the function of fecundity. The fact that the xanthophyll in the epidermis is found in a granular form shows also that the hypothesis upon which Blakeslee and Warner[3] have based their explanation of the relation between fecundity and loss of body color is built upon the wrong assumption that the pigment is found in the epidermis dissolved in fat. Similarly Warner and Edmond[12] have no basis for their conclusion that the higher fat content of the blood of laying hens is related directly to the fading of the skin color during fecundity.

Particularly conclusive evidence of the fact that fecundity prevents the excretion of xanthophyll through the skin is furnished by the fact that we found it impossible to restore the pigment to colorless birds during fecundity, even by very heavy feeding of xanthophyll-rich feeds. We also found it impossible to color the adipose tissue of laying carotinoid-free hens by heavy xanthophyll feeding. All the xanthophyll of the food apparently is deflected to the ovaries and becomes a part of the egg yolk, none becoming incorporated with the adipose or other fatty tissues.

The question is raised as to what bearing these results have upon the fact that certain breeds, like the Orpington, are hereditarily free from xanthophyll in shanks, beak, ear lobes, etc., in spite of the fact that their blood serum, body fat, and egg yolks are as rich in pigment as any of the yellow-skinned breeds, provided the food contains xanthophyll. Blakeslee[4] and his coworkers state that certain strains of Leghorns have been bred which always lack distinct yellow pigment in ear lobes, etc. It is possible, therefore, that absence of visible pigmentation may be an acquired characteristic in the case of the Orpington breed. We hope to throw some light on this point through several generations of White Leghorns bred and raised without xanthophyll in the feed.

The most important practical application of the observations reported in this paper is in clearing up considerable of the confusion which has arisen regarding the use of pigmentation of ear lobes, etc., as a criterion for judging the laying ability of hens of certain breeds. It seems clear to us that the absence of pigmentation at the end of the laying season is not necessarily a sign of high fecundity but one of continuous fecundity only, for a more or less definite period, depending upon whether it is the thin or heavy skinned parts of the body which are used for judging this fact. Hens which complete the season with yellow shanks, beaks, ear lobes, etc. have either laid intermittently or have lost their fecundity long enough for the pigment to be restored. It is, therefore, imperative that the judging of egg laying by the color of the ear lobes, shanks, etc. be done immediately after the hen begins to moult in the summer in order to prevent the observations being vitiated by a restoration of pigment through loss of fecundity.

CONCLUSIONS.

1. The fading of the yellow pigment from the ear lobes, beak, shanks, etc. of hens of the Leghorn and American breeds during fecundity is due to the fact that fecundity deflects the normal path of excretion of the xanthophyll from these parts of the skin to the egg yolk.

The xanthophyll deposited in the epidermis of the above mentioned parts gradually disappears as the result of the natural physiological change in the structure of the skin.

The thicker the epidermis the more slowly will the xanthophyll disappear.

2. The xanthophyll in the epidermis of the ear lobes, beak, shanks, etc. of Leghorn and American breeds of fowls is largely in granular form with little or no fat associated with it. It is found chiefly in the rete of Malphigi but also along the blood capillaries of the subcutaneous tissue.

3. It is impossible to restore xanthophyll to the skin of hens as long as fecundity exists no matter how large an excess of pigment is fed.

4. Adipose tissue also fails to take up the xanthophyll from the food during egg laying even on rations rich in xanthophyll, the pigment being excreted wholly in the egg yolks.

5. The fading of the ear lobes, beak, and shanks of the Leghorn and American breeds of hens as the result of egg laying is an index of *continuous fecundity only*, not of *heavy egg laying*.

Yellow color in these parts immediately after the end of the laying season indicates either intermittent fecundity or a more or less recent loss of fecundity for a period of time sufficient for the xanthophyll to be restored to the body.

We wish to thank Prof. D. H. Dolley, of the Department of Pathology of the University of Missouri, for his generous co-operation in connection with the histological studies.

EXPLANATION OF PLATES.

PLATE 3.

FIG. 1. Cock 482 raised on carotinoid-free ration, after 275 days in small exhibition cage, during which time he was colored with xanthophyll by feeding yellow corn for 42 days, after which he was allowed to lose the xanthophyll in the visible skin parts by withdrawing the xanthophyll from the food.

PLATE 4.

FIG. 2. Vertical section of highly pigmented shank skin, stained with Nile blue, showing pigment granules in corium, Malphigian layer, and along blood capillaries in corium. The fat globules, stained red by the Nile blue, are also shown.

FIG. 3. Vertical section of carotinoid-free shank skin, stained with Nile blue, showing the absence of pigment granules and the fat globules, stained red by the dye.

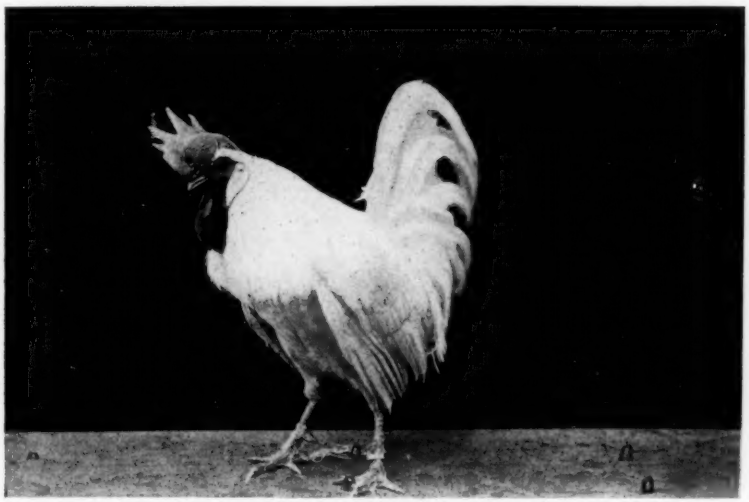

FIG. 1.

(Palmer and Kempster: Yellow pigmentation and fecundity.)

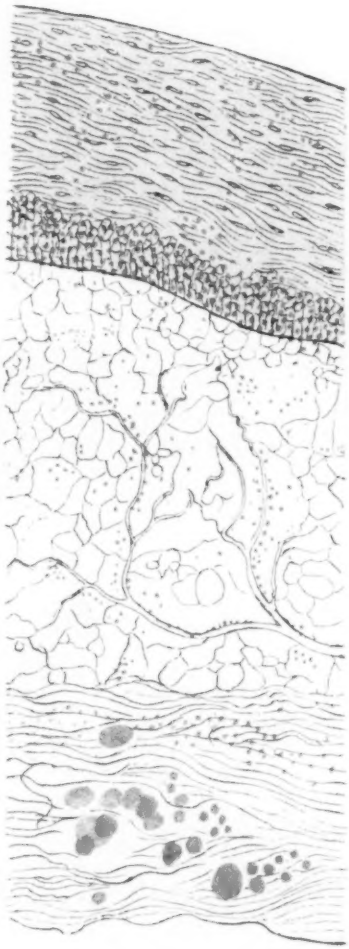

FIG. 2.

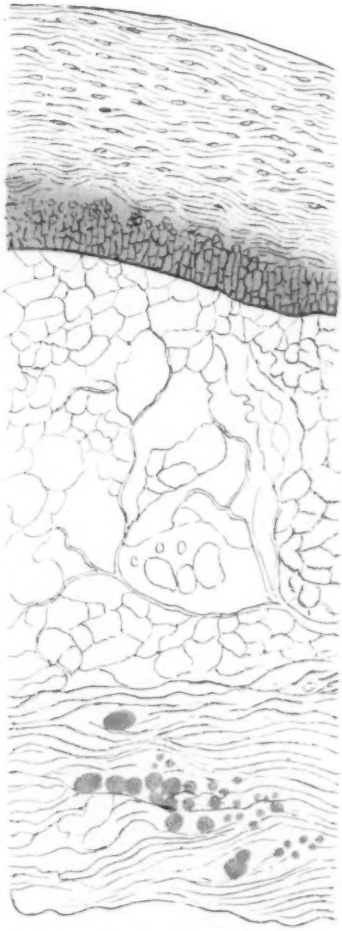

FIG. 3.

(Palmer and Kempster: Yellow pigmentation and fecundity.)

THE INFLUENCE OF SPECIFIC FEEDS AND CERTAIN PIGMENTS ON THE COLOR OF THE EGG YOLK AND BODY FAT OF FOWLS.*

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(Received for publication, July 9, 1919.)

A previous paper[1] has reported the success which was attained in raising White Leghorn chickens from hatching to maturity on rations devoid of carotinoids. This result, together with the fact that the hens in our carotinoid-free flock exhibited normal fecundity, made it possible to make a more critical study of the effect of various coloring matters upon the pigmentation of the adipose tissue, egg yolk, and visible skin parts of fowls of the White Leghorn breed than is possible with birds normally pigmented with carotinoids.

Influence of Carotin and Xanthophyll.

We were particularly interested, among other things, in confirming the observation made previously by one of us[2] that fowls use little if any carotin for the pigmentation of adipose tissue, blood serum, and egg yolk, xanthophyll being the chief plant carotinoid responsible for the yellow pigmentation normal to animals of this species.

Five of the cockerels raised on carotinoid-free rations were fattened for 2 weeks by feeding heavily with white corn and skim milk. The birds gained in weight from an average of 2.57 to an average of 3.08 pounds. Birds 400 and 345 were fed the carotin ration and Birds 480, 482, and 485, the xanthophyll ration.

* Published by permission of the Director of the Missouri Agricultural Experiment Station.

[1] Palmer, L. S., and Kempster, H. L., *J. Biol. Chem.*, 1919, xxxix, 299.

[2] Palmer, L. S., *J. Biol. Chem.*, 1915, xxiii, 261.

Influence of Carotin.—Carotin was fed in the form of naturally very highly colored butter secured from the colostrum milk of a Jersey cow. The butter was thoroughly mixed with the ration of white corn and skim milk.

At the beginning of the experiment neither bird showed any visible traces of pigmentation in the ear lobes, beak, shanks, etc. The carotin ration was continued with Bird 400 for 20 days and with Bird 345 for 26 days, after which the birds were killed and their carcasses examined for the presence of pigment. The protocols of the two examinations follow.

Bird 400.—Base of beak and shanks very faintly tinted yellow in comparison with a check bird from the carotinoid-free flock. Tissue fat apparently colorless; rendered, melted fat in 1 inch layer had color of 1.3 units yellow, 0.2 unit red with Lovibond tintometer. Blood serum contained traces of carotin. Ether extract of oil sac on base of tail had faint yellow color.

Bird 345.—Ear lobes colorless. Eye rings colorless. Base of beak faintly yellow. Shanks faintly tinted in comparison with check bird. Oil sac contained small amount of colorless oil. Blood serum contained traces of carotin. Adipose tissue faintly yellow; rendered, melted fat in 1 inch layer had color of 4.5 units yellow, 0.5 unit red with Lovibond tintometer.

Influence of Xanthophyll.—Xanthophyll was fed in the form of yellow corn, which replaced the white corn in the previous ration. None of the birds showed a trace of yellow pigmentation at the beginning of the xanthophyll feeding. The xanthophyll ration was continued with Bird 485 for 3 days, with Bird 480 for 8 days, and with Bird 482 for 42 days. Birds 485 and 480 were killed at the end of the xanthophyll feeding but Bird 482 was put back on the carotinoid-free ration. The protocols of the three birds at the close of the xanthophyll feeding are as follows.

Bird 485.—Pigment began to appear in beak and eye rings after 72 hours. On killing, the beak, eye rings, ear lobes, and shanks were distinctly yellow in comparison with check birds. Blood plasma rich in xanthophyll; tintometer reading of extracts of 5 cc. in 14 cc. volume and 1 inch layer was 10.0 units yellow, 0.2 unit red. Oil sac full of bright yellow oil. Tissue fat not examined for xanthophyll as bird had previously been used for feeding Sudan III. (See experiment with Sudan III.)

Bird 480.—Xanthophyll quite noticeable in shanks and beak on 5th day of yellow corn feeding. On 6th day yellow was distinctly visible in

eye rings, comb skin, and ear lobes. When slaughtered on 8th day all visible skin parts were quite yellow, that in the beak extending about two-thirds out from the base. Skin on body showed no pigment. Blood serum rich in xanthophyll-ether extracts of 5 cc. showing tintometer reading of 14.0 units yellow, 0.5 unit red in 1 inch layer and 13 cc. volume. Tissue fat *not* highly pigmented; tintometer reading of 1 inch layer of rendered, melted fat was 4.2 units yellow, 0.8 unit red.

Bird 482.—Bird was fed yellow corn until all visible skin parts were deeply yellow and the feathers had a smooth, creamy appearance. Pigment began to appear in body on 5th day of xanthophyll feeding. On 6th day the shanks were distinctly yellow. At the end of the 42 days feeding the color of the beak, shanks, ear lobes, and skin at base of tail feathers was measured with the Bradley color top with the results shown in Table I.

TABLE I.

Color of Visible Skin Parts after Feeding Yellow Corn.

Part of fowl.	Yellow.	Orange.	White.
	per cent	*per cent*	*per cent*
Shanks	55	25	20
Beak	55	25	20
Ear lobes	55	0	45
Base of tail	60	0	40

Influence of Sudan III and Annatto.

Experiment with Sudan III.—Cockerel 485, raised on carotinoid-free ration, and fattened for 17 days on white corn and skim milk, was fed Sudan III for 8 days. An ether solution of the dye was poured over the white corn-meal and the ether driven off leaving the corn with a bright pink color. The amount of Sudan III fed each day by this method was not estimated. However, on the 8th day of pigment feeding 0.075 gm. of Sudan III powder was mixed with the corn-meal.

There was no visible effect of the pigment feeding in any of the visible skin parts of the bird; *i.e.*, the ear lobes, beak, shanks, etc.

The bird was put back on carotinoid-free ration for 4 days after which the white corn was changed to yellow corn. 3 days later the bird was killed. Autopsy showed that the adipose tissue in the mesentery, abdominal, and crop regions was deeply tinted pink with Sudan III, as were also the bone marrow and subcutaneous adipose tissue. No trace of the dye was evident in the epidermis in any part of the body. The blood plasma and the bile were also examined for the presence of Sudan III but no pigment was found. The effect of the xanthophyll feeding on the blood plasma has been noted in connection with the experiment already reported.

Experiment with Annatto.—Cockerel 332, raised on a carotinoid-free ration from hatching, was fed annatto for 17 days. The pigment was fed in the form of an ether extract poured over white corn-meal until the corn, on evaporation of the ether, had a deep yellow color. The artificial yellow corn-meal thus prepared was fed in the form of a wet mash with skim milk.

After 17 days of annatto feeding there was no visible pigmentation of the ear lobes, beak, eye rings, vent, or shanks. The bird was then bled to death and the oxalated blood plasma and carcass carefully examined for annatto. No test for annatto could be obtained from extracts of the blood plasma or the liver. The adipose tissue was apparently colorless but the rendered, melted fat had the faint yellow color of 1.1 units yellow, 0.1 unit red in a 1 inch layer, when examined with the Lovibond tintometer. This color was not regarded to be due to the annatto.

The gizzard, small intestine, ceca, and large intestine were examined separately for annatto and a positive test was obtained in each case using the stannous chloride test on stains left on filter paper by ether extracts of the material. Especially strong tests were obtained from the contents of the gizzard and large intestine.

The significance of the failure of Sudan III to stain any but adipose tissue has been discussed in a previous paper.[3]

We regard the failure of the pigment of annatto to be resorbed as a confirmation of the observation that none of the yellow plant pigments other than xanthophyll is of significance in the pigmentation of fowls. Schunck[4] has shown that annatto is related to the carotinoids in many of its properties and states that it has been given the empirical formula, $C_{28}H_{34}O_5$.

Influence of Specific Feeds on the Color of Egg Yolk.

The normal fecundity of the hens of our flock raised on carotinoid-free rations and the resulting absence of xanthophyll from the yolks of the eggs suggested the more or less practical experiment of ascertaining the relative effect of specific feeds on the coloration of the egg yolks. At the same time we expected to ascertain whether the adipose tissue and visible skin parts of the hens would be pigmented on xanthophyll rations while the birds were exhibiting fecundity.

Fourteen laying hens were taken from the carotinoid-free flock and placed in separate small cages with sand-covered floors and

[3] Palmer, L. S., and Kempster, H. L., *J. Biol. Chem.*, 1919, xxxix, 313.
[4] Schunck, C. A., *Proc. Roy. Soc. London*, 1904, lxxii, 165.

were fed the following rations, respectively, for a period of 4 weeks. Tap water and oyster shell were kept before the birds at all times.

Ration 1.—Check ration: Baked mixture of corn-starch and skim milk powder, dried and ground.

Ration 2.—Wheat ration: Whole wheat fed as scratch feed with mash of white corn-meal. Pork liver fed at intervals and skim milk *ad libitum*.

Ration 3.—Yellow corn ration: Yellow corn as scratch feed and yellow corn-meal as mash, supplementing with pork liver and skim milk.

Ration 4.—Red corn ration: Same as Ration 3, the red corn replacing the yellow corn.

Ration 5.—Gluten feed ration: White corn as scratch feed, mash of gluten feed and white corn-meal, 1:3, supplementing with pork liver and skim milk.

Ration 6.—Oat ration: White corn as scratch feed, oatmeal as mash, supplementing with pork liver and skim milk.

Ration 7.—Barley ration: Scratch feed of barley and white corn, 1:2, mash of white corn-meal and barley meal, 1:3, supplementing with pork liver and skim milk.

Ration 8.—Cottonseed meal ration: White corn as scratch feed, mash of cottonseed meal and white corn-meal, 1:3, supplementing with pork liver and skim milk.

Ration 9.—Hemp seed ration: 25 per cent hemp seed in mash and scratch feed, the remainder being white corn-meal and white corn. Pork liver and skim milk as in other rations.

Ration 10.—Rape seed ration: Similar in every way to hemp seed ration.

Ration 11.—Green feed ration: White corn, liver, and milk, with green feed in abundance.

Ration 12.—Meat scrap ration: White corn for scratch feed, mash of white corn-meal and meat scrap, 3:1. No milk or liver.

Ration 13.—Blood meal ration: White corn for scratch feed, mash of white corn-meal and blood meal, 3:1. No milk or liver.

Ration 14.—Wheat bran ration: White corn for scratch feed, mash of white corn-meal and bran, 3:1, supplementing with pork liver and skim milk.

The egg production of the birds during the experiment and the color of the egg yolks are shown in Table II. The egg yolk colors are given for both the raw and hard boiled yolks using the Bradley color top. In the case of the yellow corn and green feed rations the color of the raw yolks was too deep to be matched with the Bradley color top. The color readings were made for these eggs with the Lovibond tintometer.

Especially interesting was the fact that the rations rich in xanthophyll had no effect on the visible skin coloring of ear lobes,

beak, shanks, etc., of the laying hens. This was also true of the adipose tissue which failed to show the effects of the heavy xanthophyll feeding, although the egg yolks were very highly colored. These results show that all but traces of the xanthophyll of the feed goes into the egg yolk during fecundity and that it is therefore impossible to color the body fat or skin of a laying hen with the plant carotinoids natural to this species of animals.

TABLE II.

Effect of Feeds on Color of Egg Yolk.

Ration.	Bird No.	Number of eggs laid.	Raw yolk.				Hard boiled yolk.			
			Yellow.	Orange.	Green.	White.	Yellow.	Orange.	Green.	White.
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carotinoid-free	2942	17	55	0	5	40	7.5	0	2.5	90.0
Wheat	2938	13	65	10	5	20	30.0	0	2.5	67.5
Yellow corn	3152	10	*				70.0	12.5	0	17.5
Red "	2906	14	60	10	5	20	37.5	0	0	62.5
Gluten feed	2909	6	80	10	0	10	27.5	0	0	72.5
Oats	2939	9	65	10	5	20	27.5	0	2.5	70.0
Barley	2910	7	70	15	5	10	35.0	0	0	65.0
Cottonseed meal	3477†	4	55	0	5	40	20.0	0	5.0	75.0
Hemp seed	3163	7	62	23	5	10	40.0	0	0	60.0
Rape "	3188	8	65	5	0	30	20.0	0	5.0	75.0
Green food	2950	15	‡				72.5	17.5	0	10.0
Meat scrap	2934	12	65	0	0	35	20.0	0	2.5	77.5
Blood meal	2943	10	60	0	0	40	12.5	0	2.5	85.0
Wheat bran	2944	10	60	0	5	35	20.0	0	2.5	77.5

* 33.0 yellow, 4.2 red with Lovibond tintometer.

† Bird died on 21st day of experiment.

‡ 47.0 yellow, 4.5 red with Lovibond tintometer.

Some interesting results were secured in egg yolk coloration with the specific feeds. The data in Table II show that yellow corn and green feed are the only common poultry feeds rich in xanthophyll. Hemp seed, barley, gluten feed, and red corn contain small amounts of xanthophyll but the remainder of the feeds contain quantities which are practically negligible as far as their effects on the color of the egg yolk is concerned.

As stated in a previous paper by one of us,[2] yellow corn is the best winter chicken food for keeping up the color of the egg yolks or for coloring the adipose tissue during fattening.

SUMMARY.

1. Carotin and the orange-yellow pigment of the annatto seed are without influence on the color of the adipose tissue of poultry.

2. Sudan III colors the adipose tissue only of non-laying fowls, and is without effect on the color of the visible skin parts.

3. Xanthophyll, fed in the form of yellow corn, has an immediate effect on the color of both adipose tissue and visible skin parts of fowls of the type of White Leghorns.

4. The relative xanthophyll content of various chicken feeds was tested separately by feeding them to laying hens raised on carotinoid-free rations and laying eggs with xanthophyll-free yolks. Yellow corn and green feed only were found to be rich in xanthophyll. A little pigment was found in hemp seed, barley, gluten feed, and red corn. Wheat, wheat bran, oats, cottonseed meal, rape seed, meat scrap, and blood meal were found to contain negligible quantities of xanthophyll.

EFFECT OF HIGH PROTEIN ACID-FORMING DIETS ON THE EXCRETION OF AMMONIA BY RABBITS.

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(Received for publication, July 12, 1919.)

INTRODUCTION.

That there are quantitative differences, at least, as to ammonia excretion between carnivora and herbivora has long been recognized.

Salkowski (8) suggested that the extreme susceptibility of rabbits to injury after the ingestion of mineral acids and the relative immunity of dogs to the same treatment are to be explained on the basis of ammonia production. Walter (11) made the generalization that man and carnivora in general are able to dispose of any excess of acids by neutralization with ammonia within the body; while in herbivora ammonia is not available for the maintenance of neutrality and thus the carbonates of the blood must be decomposed in order to regulate tissue reaction. Hallervorden (5) even denied that rabbits, and presumably herbivora in general, are able to use ammonia in the slightest degree for neutralization purposes. Winterberg (12), on the contrary, proved satisfactorily that the rabbit does excrete an increased amount of ammonia after acid ingestion and concluded that herbivora and carnivora are *qualitatively* alike in ammonia production. Eppinger and Tedesko (3) advanced experimental evidence in support of the theory that the differences in ammonia excretion between herbivora and carnivora depend entirely on the dietary habits of the two types of animals and that conditions can be reversed by altering the food. Thus the dog on a low protein diet becomes as sensitive to acid intoxication as the normal rabbit; while herbivora (sheep) on a high protein ration become as immune as the meat-fed dog. According to this view the maximum extent of ammonia production is measured by the amount of nitrogenous material undergoing metabolism. Pohl (7), working with rabbits, could not duplicate these results and hence disputed the above conclusion.

That some herbivora, at least, possess a well developed ammonia protection against acids is apparent from the work of Steenbock, Nelson, and Hart (9), who demonstrated that the ammonia excretion in cows and calves may be raised to 24 and 37 per cent respectively of the total urinary nitro-

gen by the administration of mineral acids *per os*. Growth and development were not interfered with by the acid rations during short periods. Lamb and Evvard (6) obtained normal growth and development in swine fed rations in which acid elements predominated and to which organic or mineral acids had been added. The ammonia excretion reached a maximum of 34 per cent of the total urinary nitrogen in the animals receiving the maximum amount of acid.

The highest values for urinary ammonia in rabbits on record are those reported by Bostock (1) in which a fatal dose of hydrochloric acid, accompanied with glycine ingestion, led to the excretion of 0.175 gm. of ammonia nitrogen representing 6.58 per cent of the total urinary nitrogen. Underhill and Bogert (10) point out that rabbits, in contrast to most herbivora, excrete a considerable amount of phosphorus in the urine. On an acid-forming diet (oats) rabbits may excrete more phosphorus through the kidneys than is contained in the ration. Fitz, Alsberg, and Henderson (4) have emphasized the rôle of acid phosphate excretion in the maintenance of tissue neutrality.

We might presume that in the rabbit the conversion of ammonia to urea is more complete than in other species and hence the residual ammonia fraction is very small. Bearing on this Denis and Minot (2) have obtained evidence to the effect that the sole use of ammonia in the urine is to neutralize acids formed during metabolism and that the idea of a residual ammonia fraction is superfluous.

In view of the facts mentioned above it appears desirable to determine the effect of high protein acid-forming rations on rabbits in respect to the extent of the ammonia-protecting mechanism against endogenous mineral acids.

EXPERIMENTAL.

All the rations used produced acid urines in rabbits, with the exception of one which contained sodium citrate. They may be divided into two groups: (1) low protein or control and (2) high protein, and were as follows.

Ration 1, control or low protein, consisted of carrots (moist) 50 per cent and oats 50 per cent, ground, mixed, and dried at 70–75°C. Protein = 13.65 per cent.

Ration 2 consisted of dextrin 45.8 per cent, casein 45.8 per cent, salt mixture (imitating salts of milk) 4.6 per cent, and agar 3.7 per cent. Protein = 38.2 per cent.

Ration 3 consisted of dextrin 37.1 per cent, casein 49.3 per cent, lard 4.9 per cent, salt mixture 4.9 per cent, and agar 3.9 per cent. Protein = 40.8 per cent.

Ration 4 consisted of starch 43.7 per cent, casein 43.7 per cent, salt mixture 4.4 per cent, agar 3.5 per cent, and sodium citrate 4.8 per cent. Protein = 37.9 per cent.

Ration 5 consisted of carrots (moist) 32.3 per cent, dried cod meat 32.3 per cent, dextrin 32.3 per cent, salt mixture 3.2 per cent. Protein = 45.4 per cent.

Eleven rabbits were used. All the diets appeared to be unsuited for the long continued maintenance of health and some of the animals died during the preliminary period of feeding before any urine collections had been made. It is almost certain that none of the rations was complete from a nutritive standpoint, although it is probable that the excess of acid-forming elements was the most serious objection to them. Five animals survived long enough for us to obtain observation periods from 2 weeks to 2 months during which urine collections were made. The rabbits were kept in individual metabolism cages, the urine being collected under thymol-toluene. Cages and drip pans were kept clean and washed daily in thymol-toluene. Water was supplied the animals *ad libitum*.

Total nitrogen was determined by the Kjeldahl method; ammonia by the Folin aeration method; and total acidity by a modification of the Folin method, the essential points being a rather high dilution of the sample which made the end-point more visible and the use of powdered Glauber's salt instead of potassium oxalate.

Since there was no tendency for ammonia or titratable acidity to show any essential changes as the feeding period was prolonged we shall not give the complete data, but only a few typical protocols which represent in every way the general results of the experiments.

This diet of oats and carrots as shown in Table I led to the excretion of an acid urine containing small amounts of ammonia. The addition of cabbage in small quantity, later in the period and not shown in the table, caused ammonia to disappear without bringing about any marked fall in titratable acidity, though the reaction to litmus was less intense after the cabbage was fed.

Table II shows the effect of high protein acid-forming diets on ammonia excretion. In Period 1 the energy value of the ration was probably insufficient to meet the requirements of the

animal. Protein catabolism, urinary acidity, and ammonia excretion were all relatively high. The ammonia excretion during this period showed the highest absolute values which we ever ob-

TABLE I.

Rabbit 3, Control, Young Male, Weight 1,110 Gm., Diet 1, Oats and Carrots.

Date.	Feed per day.	Urine.	Litmus reaction.	N fed.	Total urinary N.	NH_3 N.	NH_3 N.	Acidity (0.1 N).
1915	gm.	cc.		gm.	gm.	mg.	per cent	cc.
March 4.....	25	23	Acid.	0.54	0.53	2.9	0.6	13.7
" 5.....	25	19	"	0.54	0.44	5.0	1.1	14.5
" 6.....	25	18	"	0.54	0.32	8.5	2.6	10.4
" 7.....	30	14	"	0.65	0.25	4.7	1.8	7.6
" 8.....	30	20	"	0.65	0.36	1.7	0.5	—
" 9.....	30	13	"	0.65	0.26	2.6	1.0	18.7

TABLE II.

Rabbit 4, Female, Weight 1,010 Gm., Diet 5, Carrots, Cod, Dextrin, and Salts during Period 1; Diet 2, Dextrin, Casein, Salts, Agar, and Lard during Period 2.

Date.	Feed per day.	Urine.	Litmus reaction.	N fed.	Urinary N.	NH_3 N.	NH_3 N.	Acidity (0.1 N).
Period 1.								
1915	gm.	cc.		gm.	gm.	mg.	per cent	cc.
March 1.....	20	36	Acid.	1.45	0.77	11.5	1.5	16
" 2.....	20	60	"	1.45	1.28	17.4	2.1	—
" 3.....	20	66	"	1.45	1.20	35.2	2.9	—
" 4.....	20	62	"	1.45	1.13	25.3	2.2	13
Period 2.								
April 4.....	25	27	Acid.	1.63	0.52	3.3	0.6	0.3
" 5.....	25	37	"	1.63	0.72	6.1	0.9	3.9
" 6.....	25	26	Neutral.	1.63	0.34	3.8	1.1	1.5
" 7.....	25	17	"	1.63	0.22	4.9	2.2	3.7
" 8.....	25	19	Acid.	1.63	0.25	4.2	1.7	3.9
" 9.....	25	29	"	1.63	0.76	1.9	0.3	2.7

tained. Since total urinary nitrogen was also very high the *per cent* of the total nitrogen present as ammonia was not notably high. In Period 2 more food, of a higher energy value due to

the presence of the lard, was consumed. Here the protein-sparing effect of the additional calories is shown. The acidity, total nitrogen, and ammonia of the urine markedly declined even on an increased protein intake. Urinary ammonia expressed as *percentage* of the total nitrogen is, however, nearly as high as in Period 1.

The addition of sodium citrate to a high protein acid-producing diet as shown in Table III caused a marked fall in the acidity of

TABLE III.

Rabbit 5, Female, Weight 1,365 Gm., Diet 2, Dextrin, Casein, Salts, and Agar during Period 1; Diet 4, Starch, Casein, Salts, Agar, and Sodium Citrate during Period 2.

Date.	Feed per day.	Urine.	Litmus reaction.	N fed.	Urinary N.	NH_3 N.	NH_3 N.	Acidity (0.1 N).
Period 1.								
1915	*gm.*	*cc.*		*gm.*	*gm.*	*mg.*	*per cent*	*cc.*
March 18.....	18	41	Acid.	1.09	0.80	6.2	0.8	37
" 19.....	20	39	"	1.22	0.76	5.4	0.7	—
" 20.....	16	38	"	0.97	0.60	5.8	1.0	29
" 21.....	19	43	"	1.14	0.68	21.9	3.2	25
" 22.....	18	57	"	1.07	0.87	6.5	0.7	35
" 23.....	15	36	"	0.92	0.52	5.2	1.0	20
Period 2.								
March 31.....	30	129	Neutral.	1.82	0.84	4.2	0.5	15
April 1.....	30	154	Acid.	1.82	1.00	5.8	0.6	11
" 2.....	18	136	Neutral.	1.09	0.89	0.0	0.0	5
" 3.....	17	47	Alkaline.	1.05	0.67	4.3	0.6	2
" 4.....	14	78	"	0.84	1.12	4.8	0.4	0
" 5.....	20	59	"	1.21	0.85	3.5	0.4	5

the urine accompanied by a decline in ammonia excretion. These effects were shown even more strikingly in an experiment with Rabbit 4 not reported here. It thus appears that the ammonia-protecting mechanism in rabbits, slight even under the most extreme conditions, tends to approach zero when fixed bases are available in sufficient quantities, even though protein metabolism is occurring on a high level. The inefficiency of ammonia protection in rabbits is illustrated in Table IV.

Rabbit 6 was under observation about a month, most of the time becoming accommodated to the experimental diet. Though the food was taken fairly well this rabbit appeared unusually sensitive to the high protein acid-producing food and died on March 10, before the experimental period had progressed far. There was a loss of 345 gm. in weight during the last 10 days of life. In spite of the apparent need of protection against acids the excretion of ammonia did not rise sharply until the day preceding death, and even then the urinary ammonia nitrogen constituted only 2.2 per cent of the total nitrogen. It would seem that ammonia protection in the rabbit is slight and, even under extreme conditions, of no great benefit to the animal.

With the thought that possibly in the rabbit, due to some difference in protein metabolism and urea formation peculiar to

TABLE IV.

Rabbit 6, Female, Weight 1,180 Gm., Diet 2, Dextrin, Casein, Salts, and Agar.

Date.	Feed per day.	Urine.	Litmus reaction.	N fed.	Urinary N.	NH_3 N.	NH_3 N.	Acidity (0.1 N).
1915	*gm.*	*cc.*		*gm.*	*gm.*	*mg.*	*per cent*	*cc.*
March 7.....	20	55	Acid.	1.45	0.60	9.5	1.6	28
" 8.....	20	59	"	1.45	0.64	6.8	1.1	28
" 9.....	0	111	"	0.0	1.03	22.2	2.2	24
" 10.....	0	50	"	0.0	0.54	19.3	3.6	17

the species, no or little ammonia is available for neutralization purposes we tried the addition of an ammonium compound (ammonium lactate) to a milk ration. We had found that rabbits fed milk excrete an acid urine containing small amounts of ammonia. The results of a typical experiment are shown in Table V.

The addition of ammonium lactate to a milk diet in every experiment (three in all) resulted in a reduction and finally the complete disappearance of ammonia from the urine. There was a considerable increase in protein catabolism, shown by the increase in urinary nitrogen, with an initial increased acidity followed by a pronounced fall in the latter, in one case the urine becoming alkaline to phenolphthalein after the ingestion of 3 gm. of ammonium lactate at the rate of 1 gm. per day. In this case

also ammonia disappeared entirely from the urine which possessed a strong mouse-like odor not unlike that of impure acetamide. No toxic symptoms were observed after ammonium lactate ingestion. It would seem from these experiments that in the rabbit, even when ammonia is available in the blood and when endogenous acids are being produced beyond the capacity of the fixed bases present to neutralize, the acid-base regulatory mechanism does not involve the excretion of any considerable quantities of ammonium salts in the urine.

TABLE V.

Rabbit 2, Male, Weight 1,400 Gm., Diet, Milk with Addition of Ammonium Lactate as Noted.*

Date.	Milk per day.	Urine.	Litmus reaction	N fed.	Urinary N.	NH_3 N.	NH_3 N.	Acidity (0.1 N).
1915	*cc.*	*cc.*		*gm.*	*gm.*	*mg.*	*per cent*	*cc.*
July 21...						1.1		4.1
" 27...	240			1.40	0.38	1.2	0.3	8.6
" 28...	200	93	Acid.	1.16	0.40	2.2	0.6	14.9
Aug. 2...	100 + 1 gm. am. lactate.	50	"	0.69	0.79	2.9	0.2	42.9
" 3...	200 + 1 gm. am. lactate.	67	"	1.24	0.47	0.5	0.1	16.9
" 4...	As on Aug. 3.	136	Neutral.	1.24	0.64	0.8	0.1	20.8
" 6...	" " " 3.	140	"	1.24	0.73	0.0	0.0	2.3

* 1 gm. of ammonium lactate contained 0.0805 gm. of ammonia nitrogen.

SUMMARY.

Rabbits fed high protein diets in which the acid-forming elements predominate excrete a distinctly acid urine containing appreciable quantities of ammonia.

Ammonia as a factor in the acid-base equilibrium in the rabbit, in contrast to some other herbivora, *e.g.* cow, is relatively unimportant even under conditions of supposedly severe acid intoxication.

The addition of sodium citrate to a high protein acid-producing diet caused in the rabbit a marked fall in urinary acidity and, when administered in sufficient quantity, the disappearance of urinary ammonia.

When ammonium lactate in small quantity is added to a milk diet (high protein acid-producing in the rabbit) there is a fall rather than a rise in ammonia excretion, indicating that the failure of the rabbit to utilize ammonia to a greater extent for neutralization purposes is due not so much to a deficiency of ammonia in the organism as to other causes.

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ACID FERMENTATION OF XYLOSE.

By E. B. FRED, W. H. PETERSON, and AUDREY DAVENPORT.

*(Contribution from the Chemical Section, Science and Research Department, Bureau of Aircraft Production, in Cooperation with the Departments of Agricultural Bacteriology and Agricultural Chemistry, University of Wisconsin.)**

PLATE 5.

(Received for publication, July 3, 1919.)

It has been known from the earliest times that if sugar solutions, acid in reaction, are left exposed to the air, in a few days they become turbid, the sugar gradually disappears, and in approximately the same ratio, alcohol appears. In time the alcoholic liquid becomes covered with a tough mucinous skin and is gradually oxidized to acetic acid. It was Kützing (1837) who first showed that the "mother of vinegar" consists of minute organisms grouped in the form of chains. It remained, however, for Pasteur to show (1864) that this fermentation of the alcohol to acetic acid

* Early in the development of the airplane program it became evident that the chemical industries were confronted by a very large shortage of acetic acid and its derivatives, among which may be mentioned acetic anhydride, acetone, methyl acetate, amyl acetate, and some other compounds necessary to the manufacture of cellulose acetate and the various solvents used in the compounding of airplane "dopes." The manufacture of smokeless powder had absorbed and was absorbing practically all the acetone produced. In order to meet this shortage several lines of research were undertaken by this Department, in cooperation with the Bureau of Chemistry, and the description of one of these—"The acid fermentation of xylose," by E. B. Fred, W. H. Peterson, and Audrey Davenport, is herewith presented. An article descriptive of another successful development is in preparation.

It was first suggested to me by L. A. Round, of the Bureau of Chemistry, that xylose might be fermented into some valuable compounds. Since a method for the production of xylose on a commercial basis and in large quantities at a very low cost from such abundant materials as corn cobs and wood had recently been originated in the Carbohydrate Lab-

is a physiological process, bound up with the vital activity of the minute organisms termed *Mycoderma aceti*. Several years later Hansen (1879) secured pure cultures of the acetic acid bacteria and carefully described their morphology and physiology. He found that different species are involved in this acid fermentation, *Bacterium aceti*, *Bacterium pasteurianum*, and *Bacterium kützingianum*.

Since the publication of Hansen's papers, many other species of acetic acid bacteria have been described. Among these are certain organisms which ferment sugar solutions with the formation of acids directly. Several strains of organisms capable of converting sugars directly to acids will be described in this paper. Just how the sugars are broken down and what the intermediate products are we have not determined, since we have been especially interested in the end-products.

The possibility of securing acetic acid from the pentose sugar, xylose, by bacterial action was considered by the Chemical Section of the Science and Research Department of the Bureau of Aircraft Production to be sufficiently promising to warrant investi-

oratory of the Bureau of Chemistry, by C. S. Hudson, F. B. La Forge, and T. S. Harding, it was considered very advisable to attempt to develop solvents from this new source of supply.

E. B. Fred, of the University of Wisconsin, was asked to undertake the bacteriological work, and in view of the congested conditions in the Washington laboratories, cooperation was arranged with the University of Wisconsin, and the work carried on there. All the xylose was prepared by the Carbohydrate Laboratory of the Bureau of Chemistry, and Dr. Round assisted in starting the work by furnishing the information he had gathered and a culture he had isolated.

It is possible that acetic and lactic acids from xylose might have become commercial articles had the war needs continued, and the process classed among the successful developments of new fermentation processes to meet the urgent and extraordinary demands.

Thanks are due to the University of Wisconsin and the Bureau of Chemistry for generous cooperation.

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and
Chemist in Charge, Color Laboratory, Bureau of Chemistry.

gation and this work has been carried on for that Bureau. In all the investigation the production of acetic acid from this sugar has been constantly kept in mind; and by means of quantitative determinations those cultures most active in the production of acetic acid were selected for use in these experiments.

From a review of the literature, it is at once apparent that little attention has been paid to the bacterial fermentation of xylose. A number of investigators have noted that the yeasts cannot ferment xylose. It is true, a few investigators have reported the fermentation of xylose in yeast cultures, but their results are no doubt due to the presence of bacteria in the yeast culture.

Stone (1890), Fischer and Lafar (1905–07), and others found that yeasts alone are unable to ferment xylose. In 1911, Cross and Tollens confirmed the earlier observation that pentose sugars are not fermented by yeasts. In a nutrient culture medium, they found that yeasts assimilate a small amount of xylose. On the other hand, the bacteria commonly found in pressed yeast may ferment xylose with the formation of lactic acid.

In the course of an extensive investigation on the conditions surrounding the production of lactic acid by bacteria, obtained from various sources, Kayser (1894) isolated an organism from sauerkraut which fermented a number of sugars, including xylose and arabinose with the production of lactic acid. From glucose, acetic acid as well as lactic acid was obtained, but it is not clearly stated that acetic acid was also obtained from the pentoses.

According to Gayon and Dubourg (1904), xylose is the only pentose which is sensitive to the action of the "mannit-forming organism." In the presence of calcium carbonate and 5 per cent of xylose, they found the products of fermentation were acetic acid, lactic acid, and small amounts of alcohol and carbon dioxide. Müller-Thurgau and Osterwalder (1913) extended the work with the "mannit-forming bacteria." They noted a rapid fermentation of xylose by these organisms. The majority of "mannit-forming bacteria" attack xylose in much the same way, forming lactic acid and large amounts of acetic acid.

In 1898 Bertrand studied the action of the sorbosè-forming bacteria on xylose. He found that these organisms ferment xylose slowly with the formation of monobasic xylonic acid.

Bendix (1900) reported the occurrence of the xylose-fermenting bacteria in the feces of man and cows. According to Emmerling (1897) *Bacillus mycoides* ferments xylose and arabinose with the formation of small amounts of non-volatile acid. Grimbert (1896) found that the pneumobacillus of Friedländer converts xylose into alcohol, acetic and succinic acids, and a trace of lactic acid. According to Salkowski (1900), the

protein-decomposing bacteria in a rich nitrogenous medium decompose xylose with the production of the volatile acid, acetic, and the non-volatile acid, succinic. The pectin-fermenter, *Plectridium pectinovorum*, according to Störmer (1903), converts xylose into hydrogen, carbon dioxide, and acetic and butyric acids.

Reports from Stoklasa (1908) and Löhnis (1908) indicate that xylose furnishes an excellent source of carbon for the nitrogen-fixing bacteria, superior to lactose, maltose, sucrose, and many other sugars.

From this brief review of the literature on the fermentation of xylose, it will be seen that the data available are meager and contradictory.[1] The reason that xylose has received but little study is obvious. Until recently it has been classed as a rare sugar, difficult to prepare. However, improved methods of preparation by Hudson and La Forge and Harding have made it possible to secure xylose in larger quantities and at relatively low cost.

Methods.

The possible application of xylose fermentation for use in the manufacture of organic acids has been kept in mind. Accordingly search was made for organisms which possessed one or more of the following characteristics: (1) rapid fermentation with a high yield of acid; (2) growth in a medium of relatively simple composition; (3) growth in a medium of acid reaction; (4) growth at high temperatures. It is needless to say that any organism or organisms which possess these characteristics could be cultivated on a commercial scale without any great danger of contamination.

In view of the wide occurrence of the pentosans in plant tissue, the fermentation of pentoses must be a vital process common to many organisms. It was therefore supposed that substances rich in xylan, such as corn silage, sauerkraut, and manures, would contain xylose-fermenting bacteria. These substances and also soil were used to inoculate a liquid, yeast medium containing xylose and an excess of calcium carbonate. From the impure cultures which had a characteristic vinegar odor, subinoculations were made into flasks of sterile media and from the subcultures which produced the largest amounts of volatile acid dilution

[1] The complete historical review of the general subject of xylose fermentation is not given in this paper. The reader is referred to the attached Bibliography.

plates were poured with xylose-yeast agar. Colonies were fished from these plates and seeded into flasks of the liquid xylose medium, and after sufficient incubation the volatile acid was determined. By repeated subinoculations and platings many xylose fermenters, varying in their power of acid production, were obtained.

Some time was spent in determining what concentration of the pressed yeast infusion was necessary and most favorable for the growth of the xylose fermenters, and work was also done on a medium made of yeast cells dried at different temperatures. But the medium which gave good results and which seemed most practicable for this investigation was prepared as follows: 450 gm. of pressed yeast were steamed 3 to 4 hours with 4,500 cc. of tap water with occasional stirring. This infusion was then allowed to stand undisturbed until a heavy deposit of yeast cells and other suspended matter had settled to the bottom of the vessel. The portion above this deposit was carefully poured off and sterilized in liter flasks. These flasks of the sterile yeast infusion were allowed to stand for 1 to 2 weeks, when the supernatant liquid was syphoned off and used without clearing or filtering. This method of preparing the yeast infusion was used because no satisfactory method of filtering out the yeast cells could be found at that time.[2] For a few of the experiments this medium was cleared with egg albumin and a clear yellow liquid resulted. The desired amount of xylose was then added and if calcium carbonate was not to be used in the medium, its reaction was adjusted to a hydrogen ion exponent of about pH = 7.0 by titration with phenol red. Accurately measured portions were then pipetted into Erlenmeyer flasks and sterilized at 12 pounds pressure for 30 minutes. Unless otherwise stated, sterilized calcium carbonate was added in excess to the flasks of sterilized medium in order to neutralize the acidity as it was formed.

After an incubation period of from 2 to 4 or 6 weeks, the volatile acid was determined. The entire culture, approximately 50 cc., was transferred to a small Kjeldahl flask, and 20 gm. of acid sodium phosphate and 15 cc. of 85 per cent phosphoric acid were

[2] The work of C. S. Hudson (unpublished) has shown that the addition of infusorial earth permits the filtration of yeast material.

added. During distillation the volume of the liquid was kept constant by the addition of water from a dropping funnel which passed through the stopper of the distilling flask. A low flame was used and the distillation carried on for about 2 hours until 150 to 200 cc. of liquid were driven over, or until the fresh distillate gave no reaction with blue litmus paper.

At first some difficulty was experienced in obtaining uniform results. Sodium sulfate was used in place of sodium phosphate, but it was observed that this method gave extraordinarily high results at times, particularly if the volume of liquid in the distilling flask became low. Qualitative tests on the distillate showed strong tests for sulfites. To avoid this reduction of the sulfate by the organic matter of the culture, phosphates were substituted and the volume in the distilling flask was carefully controlled. Under these conditions uniform results were obtained. Some idea of the influence of the reagents and of acetic acid, lactic acid, and the media may be obtained from Table I.

TABLE I.

Volatile Acid by the Phosphoric Acid Method.

No.	Added to phosphate solution.	0.1 N volatile acid in 150 cc. of distillate.
		cc.
1		0.25
2		0.30
3	24.2 cc. 0.1 N acetic acid	24.20
4	24.2 " 0.1 " " "	23.80
5	22.2 " 0.1 " lactic "	1.40
6	22.2 " 0.1 " " "	0.60
7	50 " xylose-yeast medium	3.30
8	50 " " " + 33.3 cc. 0.1 N acetic acid	37.30
9	50 cc. xylose-yeast medium + 33.3 cc. 0.1 N acetic acid	36.80

Occurrence of Xylose Bacteria and Fermentation of Xylose in Impure Cultures.

In view of the wide distribution of xylan in nature, it is most probable that the xylose fermenters are common inhabitants of decomposing plant tissue, of soil, and of manure. Support for

this assumption is seen in the figures of Table II. 2 per cent xylose-yeast water was inoculated with small amounts of manure, soil, sauerkraut, and silage and allowed to incubate for 2 weeks

TABLE II.

Fermentation of Xylose in Mixed Cultures.

No.	Source of inoculum.	0.1 N volatile acid in 100 cc. of culture.
		cc.
1	Soil	102.2
2	"	53.5
3	"	42.9
4	"	53.4
5	"	22.8
6	"	2.3*
7	Cow manure	53.0
8	" "	53.4
9	" "	195.1
10	Manure enrichment	15.8
11	" "	21.0
12	" "	14.0
13	" "	42.2
14	Corn silage	48.4
15	" "	75.4
16	" "	79.2
17	" "	52.2
18	" "	72.4
19	" "	116.2
20	" "	191.6
21	" "	190.9
22	Alfalfa "	82.0
23	" "	123.0
24	" " subculture	128.4
25	" " "	119.6
26	" " "	99.6
27	Sauerkraut	47.4
28	"	44.2
29	"	30.8

* Molds.

or more at 30°C. In many of the flasks, especially the cultures inoculated with manure, silage, and soil, a profuse growth of mycodermæ and molds was noted. Wherever these organisms developed in large numbers, forming a heavy gelatinous film, the

yield in volatile acid was low. In nearly all these impure cultures microscopic mounts showed the presence of mycodermæ, yeasts, short rod forms, and often molds. The majority of cultures, 1 week after inoculation, were marked by a strong fruity odor; in some the characteristic odor of vinegar predominated. From the odor of the cultures, an approximate idea of the acetic acid content could be gained; *i.e.*, cultures which smelled strongly of acetic acid always gave a large amount of volatile acid. With some of the cultures, transfers were made every 2 days into flasks of the same medium in order to eliminate the mycodermæ or to favor the growth of volatile acid-forming organisms, but volatile acid production was not stimulated in the subcultures. The point of interest in connection with Table II is the general distribution of the xylose fermenters in nature; they were found in manure, soil, sauerkraut, and silage.

Isolation of Xylose Bacteria and Fermentation of Xylose in Pure Culture.

From the cultures which gave a large yield of volatile acid, plates were poured with xylose-yeast-agar and incubated at 30°. After 24 to 48 hours the plates were examined, and according to microscopic mounts made from several colonies, only two types of organisms were present, one a mycoderma, and the other a rod either singly or in chains. Many colonies were fished from these plates and seeded into tubes of liquid 2 per cent xylose-yeast medium.

More recent work has shown that pure cultures of xylose fermenters may be obtained by plating directly, without a preliminary growth in liquid cultures. In the selection of the acid-forming organism it was noted that small colonies, often deep in the agar, gave the best results. Both methods of isolation were used and in this way several hundred pure cultures obtained. The acid production of these various cultures was studied and from those strains which gave the highest yield in volatile acid, pure cultures were selected for further study. The pure cultures isolated in this way were seeded into flasks of 2 per cent xylose-yeast-liquid medium and incubated for 2 weeks at 30°. At the end of the incubation period all the cultures were analyzed for

total volatile acid. The majority of the cultures produced little if any acid and were therefore discarded. Twenty-four, however, formed appreciable quantities of volatile acid which gave positive tests for acetic. To establish the kind of volatile acid formed, colorimetric qualitative tests were applied to the distillates con-

TABLE III.

Fermentation of Xylose in Pure Cultures.

No.	Culture No.	Source of cultures.	0.1 N volatile acid in 100 cc. of culture.
			cc.
1	20–5	Corn silage.	70.7
2	24–1	" "	108.4
3	25–1	" "	104.4
4	41–11	Field soil.	148.4
5	41–12	Corn silage.	58.0
6	55–9	" "	158.2
7	66–2	" "	42.4
8	66–3	" "	33.6
9	66–5	" "	60.0
10	69–3	" "	137.6
11	69–8	" "	148.4
12	69–10	" "	132.8
13	69–13	" "	134.0
14	69–19	" "	134.4
15	69–23	" "	146.0
16	69–26	Sauerkraut.	115.6
17	69–27	"	138.8
18	69–30	"	147.8
19	69–32	"	122.4
20	118–2	Horse manure.	147.2
21	118–4	Cow "	144.4
22	118–5	Hog "	154.6
23	118–8	Sheep "	143.0
24	118–13	" "	150.0
25	487–1*	Sauerkraut.	138.9

* Culture from L. A. Round.

taining the volatile acids. For this purpose, the distillates were concentrated on the steam bath to a small volume and then tested for the various volatile acids as outlined by Dyer (1916–17). In every case a very strong test was obtained for acetic acid, but in no case was there any suggestion of the presence of the homologues of acetic acid. Table III gives the results of the analyses

of the twenty-four cultures which showed the highest volatile acid production.

The very general distribution of the xylose fermenters is strongly indicated by the figures of this table; twenty-four cultures out of about 150 gave a decided acid fermentation. Manure, silage, and sauerkraut contain great numbers of acid-producing organisms.

The effect of temperature of incubation, as well as time of incubation, on the yield of volatile acid has been considered. From the results of many tests it was concluded that about 30–35° gave the largest amount of acid.

In relation to time the fermentation of xylose takes place very rapidly as compared to the acetic acid fermentation of alcohol. In a 2 per cent xylose-yeast-liquid medium the maximum amount of acid is reached in about 12 to 14 days after inoculation. Additional data are presented in Table XVIII.

All attempts to break down xylose in the usual yeast-water medium by means of pure cultures of yeast or by certain of the common forms of soil bacteria, *Bacillus mycoides* and *Bacillus subtilis*, gave negative results. The value of xylose for the growth of molds has not received study. This phase of the fermentation problem deserves careful investigation.

Morphology and Physiology of Xylose Fermenters.

Morphological Characters.

The xylose fermenters are rods with blunt ends. Their length is usually two to three times their width, although filaments, sometimes very long, occur at certain stages of their growth. The size of the organism varies somewhat with the different strains. Cultures 487-1, 41-11, 55-9, 69-19, and 118-5 are about 0.6 μ wide by 1.6 to 1.8 μ long. Culture 69-30 is about 0.7 μ wide by 2.0 to 2.5 μ long, while Culture 118-8 measures about 0.7 μ wide by 2.0 to 3.0 μ long. Spores are not formed. The organisms are apparently slowly motile, although all attempts to stain flagella failed. They are Gram-positive and stain easily with the ordinary aniline dyes.

Cultural Characters.

Agar Plates.—After 24 hours on yeast-water-xylose-agar at 30° the colonies are scarcely visible to the naked eye. After 3 days the colonies are about 1 mm. in diameter and fail to show a noticeable increase in size from longer periods of incubation. The colonies are punctiform, the surfaces smooth, and the edges entire. On beef-peptone-agar growth is much slower than on yeast-water-xylose-agar.

Gelatin Stab.—Gelatin is not liquefied. Growth is uniform along line of inoculation from top to bottom.

Agar Stabs, Xylose-Yeast Medium.—Growth uniform along line of inoculation, scanty on the surface.

Agar Slants, Xylose-Yeast Medium.—Growth scanty, beaded, glistening.

Agar Shakes, Xylose-Yeast Medium.—Turbidity throughout the culture medium; no gas.

Xylose-Yeast-Water.—No surface growth. Medium moderately to strongly clouded. After 48 hours at 30° the turbidity of the medium begins to disappear and a precipitate is formed. The different strains show a slight variation in clouding.

Beef-Peptone-Bouillon.—Clouding is much slower than in the xylose-yeast-water medium.

Milk.—Litmus and bromocresol purple milk show no change after 2 weeks incubation at 30°. Apparently these organisms do not grow in milk, or if so, very slowly.

Nitrate Broth.—Nitrates are not reduced. Growth is scanty.

Cabbage Juice-Xylose Solution.—Strong clouding without gas.

Peptone-Xylose-Liquid Medium.—Growth with strong clouding.

Beef-Extract-Xylose-Liquid Medium.—Growth with moderate clouding.

Gelatin-Xylose-Liquid Medium.—Faint growth.

Asparagine-Xylose-, Urea-Xylose-, and Ammonium Sulfate-Xylose-Liquid Media.—Growth very scant or absent.

Fermentation of Carbohydrates and Related Compounds.—Acid is formed from sucrose, glucose, lactose, levulose, and mannitol in yeast-water.

Fermentation Tubes.—No gas is found in closed end of tubes. These organisms no doubt produce very small amounts of carbon dioxide, but not enough to show gas in a fermentation tube.

Reduction of Methylene Blue.—In xylose-yeast-liquid medium reduction of methylene blue is slow. About 24 hours is the time required for a test-tube culture to decolorize four drops of a 1: 10,000 methylene blue solution. The individual cultures show a variation in their time of reduction; 487–1 reduces very slowly, while 69–19 reduces faster than any of the other cultures. In every case reduction began at the bottom of the tube and extended almost to the top.

Thermal Death-Point.—In xylose-yeast-liquid medium the organisms are killed at a temperature between 55 and 60°C. for 10 minutes.

Optimum Temperature.—About 30 to 35°C. is favorable for the growth of the xylose fermenters.

Reaction.—The fermentation of xylose in yeast-water solutions stopped at a hydrogen ion concentration of about pH = 2.8 to 3.0 acid range and 8.5 to 9.0 alkaline range.

The optimum hydrogen ion concentration is about pH = 5.6.

Oxygen Relation.—The organisms are facultative anaerobes.

According to the chart of the Society of American Bacteriologists, their group number is 222.2223002. In the new classification of this Society these organisms would fall in the family Lactobacillaceæ. Although these organisms are closely related to the "mannit bacteria" described by Gayon and Dubourg in that they possess the power of forming mannitol from fructose, we feel that the most striking characteristic of the group is its power to ferment the pentose sugars. Therefore we recommend the name *Lactobacillus pentoaceticus, n. sp.*

Products of Fermentation of Xylose by Pure Cultures.

As shown in the figures of Tables II and III, xylose ferments readily in a yeast-water extract with the formation of large amounts of volatile acid which, according to qualitative tests, is made up largely of acetic acid.

Because the growth of the xylose fermenters in agar and liquid media failed to furnish any distinct differences, the cultures picked for more detailed work were chosen according to their source of isolation. It was thought that bacteria from widely separated sources might exert marked differences in their action on xylose. That this supposition is not correct will be seen from the results of later work. To study the kinds of acid or acids formed, as well as the other products produced from xylose, Cultures 41–11, 55–9, 69–19, 69–30, 118–5, 118–8, and 487–1 were inoculated into xylose-yeast-liquid medium containing known amounts of xylose.

As a rule, about 2 to 4 per cent of xylose and an excess of calcium carbonate were employed. After 2 to 3 weeks incubation, the cultures were analyzed for unfermented xylose and for volatile and non-volatile acids. The nature of the acid produced was also studied.

To determine the unfermented xylose in the cultures, the O'Sullivan-Defren method was used. The reducing ratio of glucose and xylose has never been determined by this method. The value calculated from the papers of Brown, Morris, and Millar (1897), and Daish (1914), in which the method is very similar to the O'Sullivan-Defren method, gives a result almost identical

with that of Browne, who found the ratio to be 0.983 with the Allihn method. We have therefore used the factor 0.983.

Acetic Acid.—In order to determine whether the volatile acid found by distilling the culture with sodium phosphate and phosphoric acid was entirely acetic acid or a mixture of volatile acids, the distillates obtained from Cultures 69–19, 69–30, 118–5, and 118–8 were subjected to a Duclaux distillation. The complete data obtained in one instance, 118–8, are given in Table IV. By the phosphoric acid method the aliquot used for analysis gave 73.3 cc. of 0.1 N acid. This was evaporated below 100 cc., 7.5 cc. of 1 N H_2SO_4 acid were added, and the volume was made up to 110 cc. and distilled according to the Duclaux procedure. The results are given in Table IV.

The agreement between the distilling constants found for the various cultures and that given by Duclaux for pure acetic acid is very close—as close as that found by investigators of the Duclaux method for purified acetic acid itself. In order to show that the volatile acid produced by the other cultures is acetic, the distilling constants obtained from the seven cultures chosen for a detailed study are given in Table V.

In the first three examples given in this table the Duclaux procedure was applied to the volatile acids obtained by steam distillation at constant volume as described by Dyer. In the last four cases the volatile acids were driven out of the culture solution by the use of the phosphoric method previously described. The uniform agreement between the constants found and that required for acetic acid would seem to prove conclusively that the volatile acid produced by these organisms is acetic alone. This appeared to us so unusual that we determined to check up the results obtained with the Duclaux procedure in two ways: (1) by the distilling constants obtained in Dyer's constant volume distillation and (2) by preparing the barium salts of the volatile acids and determining their barium content.

Since the second of these is an old and well recognized method for establishing the purity of a salt, it will be given first. The barium salts of the volatile acids were formed either by the titration of the distillates from the Duclaux or the Dyer distillation. The entire distillate or aliquot of the distillate was evaporated to dryness on a steam bath, taken up with water, filtered

TABLE IV.

Analysis of Culture 118–8 by the Duclaux Method.

Fraction.	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
Individual fractions of 0.1 N, *cc*...	4.48	4.77	4.86	5.10	5.36	5.64	6.06	6.62	7.45	9.02
Total 0.1 N, *cc*.....	4.48	9.25	14.11	19.21	24.57	30.21	36.27	42.89	50.34	59.36
Distilling constant, *per cent*.....	7.5	15.6	23.7	32.2	41.3	50.8	61.1	72.2	84.7	100
Duclaux constant for acetic acid..	7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100
Difference.....	+0.1	+0.4	+0.3	+0.2	+0.4	+0.3	+0.5	+0.3	+0.3	

to remove any barium carbonate resulting from an excess of barium hydroxide in the titration, and again evaporated to dryness in a weighed platinum dish. The dish and salts were then dried in an electric oven at 130° to constant weight. The barium salts of the organic acids were converted into barium sulfate by the addition of an excess of sulfuric acid, ignited, and then weighed. The weight of barium sulfate found was compared with the weight of barium sulfate calculated on the assumption that the organic barium salt was barium acetate. The close agreement between the weight of barium sulfate found and that calculated indicates that the barium salt of the volatile acid is barium acetate and corroborates the results obtained by the Duclaux analysis.[3]

TABLE V.

Distilling Constants of the Volatile Acids Obtained by the Duclaux Method.

Fraction.	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
Culture 41–11	7.6	15.7	24.1	32.8	41.9	51.6	61.8	72.9	85.2	100
" 487–1	7.4	15.3	23.4	31.9	40.8	50.2	60.2	71.1	83.2	100
" 55–9	7.6	15.4	23.6	32.0	40.9	50.3	60.3	71.1	83.8	100
" 69–19	7.4	15.3	23.5	32.3	41.4	51.7	61.2	72.3	84.8	100
" 69–30	7.5	15.4	23.8	32.6	41.7	51.2	61.5	72.6	85.6	100
" 118–5	7.6	15.5	23.8	32.3	41.4	51.0	61.2	72.4	84.9	100
" 118–8	7.5	15.4	23.0	32.3	41.3	50.9	61.1	72.1	84.7	100
Duclaux constant for acetic acid	7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100

Table VI gives the results of these determinations and the quantity of acetic acid in 100 cc. of culture as calculated from the barium salts.

In separating the volatile acids by steam distillation, the method described by Dyer for the determination of volatile

[3] As far as we know, no one has combined the preparation and analysis of the barium salts of the volatile acids obtained in the Duclaux method with the data obtained by the distillation itself. This requires but little additional work and may be a valuable check on the data obtained by the Duclaux analysis. Incidentally, the foregoing data have a bearing on the question of the value of the Duclaux method which has recently been discussed in several papers. Using the precautions for heating the distilling flask that have been advised, we have had no difficulty in checking the Duclaux figures for the distilling constant of acetic acid.

acids was followed in three cases. The culture was made up to a definite volume and centrifuged to throw down the calcium carbonate and other suspended matter. The clear supernatant liquid was decanted and a definite aliquot taken for analysis. This quantity was acidified to Congo red paper with sulfuric acid, made up to 150 cc., and distilled with steam by means of the arrangements for heating and generating a uniform current

TABLE VI.

Acetic Acid Produced by Xylose Fermenters.

Culture No.	Xylose.	Volume of culture represented by aliquot.	Volatile acids as barium salts.	Barium sulfate equivalent of volatile acids.		Acetic acid in 100 cc. of culture.
				Found.	Calculated.	
	per cent	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
Aerobic:						
118–5	—	—	0.6728*	0.6136	0.6147	—
A118–8	—	—	0.7415*	0.6755	0.6775	—
C118–8	—	—	0.6863*	0.6250	0.6271	—
69–19	2	15.0	0.1908	0.1709	0.1744	0.5859
69–30	2	15.0	0.1822	0.1634	0.1665	0.5600
118–5	2	15.0	0.1666	0.1494	0.1522	0.5256
118–8	2	15.0	0.1762	0.1590	0.1610	0.5452
69–19	3	12.5	0.2694	0.2430	0.2462	1.0000
118–8	3	12.5	0.2638	0.2382	0.2410	0.9800
41–11	4	15.0	0.3557	0.3224	0.3250	1.1054
487–1	4	15.0	0.3086	0.2786	0.2820	0.9552
55–9	4	15.0	0.4034	0.3616	0.3686	1.2398
41–11	5	10.0	0.1460	0.1302	0.1334	0.6696
Anaerobic:						
41–11	2	3.0	0.0480	0.0434	0.0442	0.7440
487–1	2	3.64	0.0520	0.0460	0.0477	0.6504
55–9	2	12.0	0.1961	0.1761	0.1792	0.7547

* Barium salts obtained from the Duclaux distillate.

of steam suggested by the author. The distillate was collected in 100 cc. portions and titrated with 0.1 N barium hydroxide. The volume in the distilling flask remained fairly constant throughout, and at the end varied not more than 10 cc. from the 150 cc. volume. In our limited experience, this distillation was more difficult to control than the Duclaux and did not give so satisfactory results. In Table VII are given the distilling constants

obtained for the three cultures together with the constant for acetic acid obtained by Stein (1913), Dyer, and ourselves. The values obtained for acetic acid and for the three cultures agree better with Stein's figures than with those given by Dyer. There is fair agreement between the constants of the cultures and that for acetic acid, but the variation is much greater than in the

TABLE VII.

Analysis of Cultures by Dyer's Distillation Method.

Fraction.	Distilling constants.					
	Culture 41-11.	Culture 487-1.	Culture 55-9.	Acetic acid.		
				Authors.*	Dyer.	Stein.†
100	30.1	33.5	32.1	33.6	30.75	33.1
200	52.5	56.0	54.5	55.6	52.04	54.5
300	68.0	69.7	68.9	70.5	66.79	68.2
400	78.5	79.6	79.0	79.9	77.00	77.6
500	85.6	85.7	85.6	86.1	84.07	84.5
600	90.2	89.5	89.7	90.3	88.97	89.6
700	93.2	91.9	92.2	93.1	92.36	93.3
800	95.1	93.7	93.8	95.3	94.71	—
900	96.4	94.9	95.0	96.6	96.34	97.4
1,000	97.2	95.8	95.8	97.4	97.47	—
1,100	97.9	96.4	96.4		98.25	99.2
1,200	98.4	97.0	96.9		98.79	99.6
1,300	98.8	97.3	97.4		99.16	99.9
1,400	99.2	97.7	97.7		99.42	
1,500		98.1	98.0			
1,600		98.4	98.3			

* Average of two determinations.

† In several fractions Stein collected 0.1 to 0.2 cc. more than 100 cc. of distillate. This departure from even hundreds would be less, however, than the difference between duplicate determinations.

case of the Duclaux distillation or in the analysis of the barium salts. In view of the more satisfactory results obtained by the two latter methods, the Dyer distillation was abandoned and the subsequent steam distillations were conducted without any attempt to maintain a constant volume or to collect separate fractions. The distillation was continued until 100 cc. of distillate required a titration of less than 0.40 cc. of 0.1 N barium hydroxide.

Lactic Acid.—An ether-water extract of the cultures gave strong positive ferric chloride, Uffelmann, and thiophene tests for lactic acid. To determine quantitatively the production of lactic acid, the residue from the steam distillation was placed in a Kutscher and Steudel extraction apparatus and extracted with ether for 50 to 75 hours. To the ether extract were added about 30 cc. of water, the ether was carefully distilled, and the water solution of the extracted acid titrated with barium hydroxide. An excess of about 5 cc. of 0.1 N barium hydroxide was added and the solution boiled for 15 minutes to convert all the lactic acid into its barium salt. The excess of barium hydroxide was titrated with sulfuric acid, phenolphthalein being used as the indicator. After standing several hours on the steam bath the barium sulfate was removed by filtration, the filtrate and the washings were evaporated to dryness, and again taken up with water. The solution was filtered to remove traces of insoluble organic matter or carbonate and the filtrate made up to a definite volume, 50 or 100 cc., and a 10 or 20 cc. aliquot was taken for analysis. In the early part of the work, this aliquot was tested for succinic acid by making it up to 100 cc. with 95 per cent alcohol, but in no case was there any precipitate formed even after standing several days. Moreover, after the aliquot was evaporated to dryness and taken up with 60 per cent alcohol, the barium salts were completely soluble. In all the later work the aliquot was therefore evaporated to dryness in a platinum dish on a steam bath and dried at 130° to dehydrate the barium salts until constant weight was secured. An excess of sulfuric acid was added and the salts were converted into barium sulfate by ignition and then weighed. From the weight of barium sulfate the corresponding weight of lactic acid was calculated. From the weight of the dried barium salts the theoretical weight of barium sulfate was calculated and thus it is shown that these salts are barium lactate. The results are presented in Table VIII.

Zinc Salts of Lactic Acid.—The zinc salts of lactic acid have been used by a number of investigators to determine both the quantity and the form of acid produced. The zinc lactate was prepared by adding slightly less than the calculated amount of 0.1 N zinc sulfate to the solution of barium lactate. After the solution was boiled for 10 minutes, 5 cc. were withdrawn and

centrifuged, and the clear supernatant liquid was tested for unprecipitated barium. The sample was then returned to the beaker, more zinc sulfate was added, if necessary, and the solution was again tested. After three or four trials the required amount, without an excess of zinc sulfate, can be determined. As a further means of determining that the proper amount of zinc sulfate has been added, a small portion may be centrifuged and tested for an excess of zinc sulfate with barium hydroxide. The solution, after standing over night, was filtered to remove the barium sulfate and the filtrate and washings were evaporated on a steam bath until crystals began to form. The dish was then

TABLE VIII.

Lactic Acid Produced by Xylose Fermenters.

Culture No.	Xylose.	Volume of culture represented by aliquot.	Weight of barium salts.	Weight of barium sulfate.		Lactic acid in 100 cc. of culture.
				Found.	Calculated.	
	per cent	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
Aerobic:						
69–30	2	15.0	0.1750	0.1290	0.1295	0.6655
118–8	2	30.0	0.3884	0.2846	0.2871	0.7319
41–11	4	7.5	0.1977	0.1453	0.1463	1.4947
487–1	4	7.5	0.1836	0.1352	0.1359	1.3906
55–9	4	7.5	0.2315	0.1700	0.1713	1.7493
41–11	5	20.0	0.2960	0.2170	0.2190	0.8371
Anaerobic:						
41–11	2	19.83	0.3084	0.2284	0.2282	0.8885
487–1	2	25.00	0.2318	0.1709	0.1715	1.0548

placed in a desiccator over sulfuric acid for 1 or 2 days until crystallization was complete and the crystals were transferred to a hardened filter paper and washed under gentle suction with 1 or 2 cc. of cold water. From the mother liquor and washings four additional crops of crystals were obtained. After drying over night in a desiccator the crystals were removed to a weighing bottle and dried to constant weight over sulfuric acid. The percentage of lactic acid developed in three cultures is given in Table IX.

The usual form of lactic acid produced in the spontaneous fermentation of sugar solutions is the racemic type. The water

of crystallization contained in the zinc salt is one means of determining the form of acid produced. The racemic zinc lactate crystallizes with three molecules of water and the active zinc lactate with two molecules, constituting 18.17 per cent and 12.9 per cent, respectively, of the weight of the salts. This water of crystallization can be driven off by heating for 2 to 3 hours at 105–108°. The water of crystallization of zinc lactate was determined in the case of the three cultures. The results obtained are shown in Table X.

TABLE IX.

Lactic Acid as Determined by the Zinc Salt Method.

Culture No.	Volume of culture represented by aliquot.	Weight of zinc lactate.	Lactic acid per 100 cc. of culture.
	cc.	*gm.*	*gm.*
41–11	26.25	0.5464	1.2601*
487–1	26.25	0.5108	1.1780
55–9	26.25	0.6260	1.4451

* The zinc salt method is known to give low values due to the difficulty of preparing and removing all the zinc salt from solution. For this reason the percentages of lactic acid per 100 cc. of culture are lower than those given in Table VIII.

TABLE X.

Water of Crystallization Contained in Zinc Lactate.

Culture No.	Weight of zinc lactate used.	Water lost.		Water in $Zn(C_3H_5O_3)_2+3H_2O$.
	gm.	*gm.*	*per cent*	*per cent*
41–11	0.4860	0.0844	17.4	18.17
487–1	0.4670	0.0840	18.0	18.17
55–9	0.6004	0.1086	18.1	18.17

It is evident from an examination of this table that the form of lactic acid produced by these organisms is the racemic type. The weight of zinc lactate used represents almost the total amount secured by the five crops of crystals and consequently would contain any impurities that separated with the crystals. A determination on the recrystallized salt would undoubtedly show even better agreement with the theoretical figure.

If the fermentation of xylose proceeds according to the following equation:

$$\underset{150}{C_5H_{10}O_5} = \underset{60}{C_2H_4O_2} + \underset{90}{C_3H_6O_3}$$

561.8 calories. 217.5 calories. 329.7 calories.

the ratio of the acetic acid to the lactic is 60 : 90, or 40 per cent and 60 per cent, respectively. It is improbable that the reaction goes quantitatively to these products without the formation of small amounts of other products, such as carbon dioxide and alcohols, but that these are of minor significance is very probable. Actually the process is not so simple as represented by the equation. A certain quantity of the xylose is most probably consumed by the bacteria for their vital functions and, consequently, the yield of acetic and lactic acids obtained is less than the theoretical quantity calculated from the equation. In Table XI are given the quantities of acetic and lactic acids produced by the seven different xylose fermenters under aerobic and anaerobic conditions and with different concentrations of sugar. The data are calculated from the barium salts of the two acids recorded in Tables VI and VIII and are given in Table XI. This table contains the most important data on the fermentation of the xylose and brings out the following significant facts.

1. The chief products resulting from the fermentation of xylose are acetic and lactic acids. Of the xylose consumed, 90 to 95 per cent is accounted for by these two substances.

2. The ratio of acetic to lactic acid is about 42 : 58, and approaches very near to the theoretical figure given in the preceding equation, 40 : 60. The lactic acid determination probably gives a somewhat low value, due to the difficulty of extracting all traces of it with ether. Were it possible to recover this completely, an even closer approximation to the theoretical ratio could be obtained, and a higher percentage of the sugar accounted for by these two products.

Minute quantities of carbon dioxide were obtained from several cultures and an examination of the distillates from several other cultures showed only slight traces of alcohol.

The question regarding what intermediate products are formed in the breaking up of the sugar molecule is of interest, but because of lack of time and the necessity of first establishing the nature of the end-products no attempt has been made in this direction.

TABLE XI.

Relative Quantities of Acetic and Lactic Acids Produced and Their Relation to the Xylose Consumed per 100 Cc. of Culture.

Culture No.	Xylose.	Acetic acid.	Lactic acid.	Total acid.	Xylose fermented.	Xylose represented by acetic and lactic acids.	Ratio of acetic to lactic.
	per cent	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	
Aerobic:							
69–19	2.00	0.5859	0.7164*	1.3023	1.489	88	45:55
69–30	2.00	0.5600	0.6655	1.2255	1.460	84	45:55
118–5	2.00	0.5256	0.7092*	1.2348	1.452	85	43:57
118–8	2.00	0.5452	0.7319	1.2771	1.410	91	43:57
69–19	3.00	1.0000	1.3284*	2.3284	2.767	84	43:57
118–8	3.00	0.9800	1.3190*	2.2990	2.670	86	43:57
41–11	4.00	1.1054	1.4947	2.6001	2.902	90	42:58
487–1	4.00	0.9552	1.3906	2.3458	2.635	90	41:59
55–9	4.00	1.2398	1.7493	2.9891	3.258	92	42:58
41–11	5.00	0.6696	0.8371	1.5067	†	—	44:56
Anaerobic:							
41–11	2.00	0.7440	1.0550	1.7990	1.900	94	41:59
487–1	2.00	0.6504	0.8885	1.5389	1.642	94	42:58
55–9	2.00	0.7547	0.9360*	1.6917	1.871	90	45:55

* Calculated from the direct titration of the extracted acid; in all other cases the figure is obtained from the barium salts.

† The determination of unfermented sugar was lost.

Effect of Certain Factors on Xylose Fermentation.

Oxygen Supply.—Several tests were made of xylose fermentation in cultures submitted to different oxygen tensions. For the first experiment it was arranged to have the same organism ferment in deep and in shallow layers. The shallow layers were prepared by pouring 100 cc. of culture medium into a 1,000 cc. Erlenmeyer flask; the deep layers, by pouring 100 cc. of culture medium into a 150 cc. Erlenmeyer flask. At the time of incubation sterilized bromocresol purple was added. Every 2 days sterilized N sodium hydroxide was added until the cultures were approximately neutral. From the results given in Table XII it is clear that shallow or deep layers, within a certain range, have little effect on the production of acid.

A repetition of the preceding experiment was made in which

calcium carbonate instead of sodium hydroxide was used as the base. One-half the cultures were incubated in an anaerobic jar and the other half as usual. From time to time, the anaerobic jar was evacuated by means of a pump, and pyrogallic acid and sodium hydroxide were added. However, it was not possible to maintain an even vacuum during the period of incubation. It will be seen from the data of Table XIII that incubation in an anaerobic jar has no harmful effect on acid production from xylose.

A similar experiment was made in which anaerobic conditions were obtained by covering the cultures with a 2 inch layer of paraffin oil. Here again the results confirm the previous tests: oxygen supply within wide limits has little effect on the activity

TABLE XII.

The Fermentation of Xylose in Deep and in Shallow Layers, after 14 Days at 30°.

No.	Culture No.	Treatment.	0.1 N acid in 100 cc. of culture.	
			Total.	Volatile.
			cc.	cc.
1	41–11	Shallow layer.	230.0	110.96
2	41–11	" "	230.0	113.92
3	41–11	Deep "	230.0	111.72
4	41–11	" "	230.0	113.12

of the xylose organisms. The figures of Table XIV support this statement. Although it was found that the oil-covered cultures made a slower growth than the uncovered ones, this difference, however, is hardly noticeable at the end of 4 weeks except in Culture 487–1.

Concentration of Xylose.—The effect of varying amounts of xylose on the production of volatile acid is given in Table XV. From the figures of the column "volatile acid," it is plain that xylose in yeast-liquid medium in amounts greater than 2 to 3 per cent fails to give a corresponding gain in acid. It seems that 2 per cent of xylose or less will give the maximum production of acid to each unit of sugar used. It is possible that with larger amounts of xylose the by-products of fermentation retard the process.

TABLE XIII.

Effect of Oxygen on the Fermentation of Xylose.

No.	Culture No.	Relation to oxygen.	0.1 N volatile acid in 100 cc. of culture.
			cc.
1	487–1	Aerobic.	114.6
2	487–1	"	114.0
Average			114.3
3	487–1	Anaerobic.	120.6
4	487–1	"	119.0
Average			119.8
5	41–11	Aerobic.	126.6
6	41–11	"	126.8
Average			126.7
7	41–11	Anaerobic.	129.8
8	41–11	"	130.6
Average			130.2

TABLE XIV.

Effect of Oxygen on the Fermentation of Xylose. Temperature 30°.

No.	Culture No.	Relation to oxygen.	0.1 N volatile acid in 100 cc. of culture.	
			After 2 wks.	After 4 wks.
1	487–1	Aerobic.	130.6	141.4
2	487–1	Anaerobic.	87.8	126.4
3	41–11	Aerobic.	130.6	131.4
4	41–11	Anaerobic.	118.6	132.4
5	55–9	Aerobic.	Lost.	134.4
6	55–9	Anaerobic.	122.8	140.4
7	69–26	Aerobic.	138.6	130.4
8	69–26	Anaerobic.	117.8	137.4

TABLE XV.

Effect of Concentration of Xylose on Fermentation with Culture 487–1, after 14 Days at 30°C.

No.	Xylose in 100 cc.	0.1 N volatile acid in 100 cc. of culture.
	gm.	*cc.*
1	2	131.4
2	2	134.8
3	3	169.6
4	4	189.6
5	5	174.4
6	6	172.0

A second experiment was made. The results are recorded in the figures of Table XVI. Although the figures show great variations, it is believed that the results are comparative and at least give an index of the effect of concentration on fermentation. In agreement with the previous experiment, it was found that about 3 to 4 per cent of xylose gave the maximum yield, while about 2 per cent gave the largest amount of volatile acid in proportion to the sugar. In amounts greater than 4 per cent of xylose there was an actual decrease in the total acidity formed.

TABLE XVI.

Effect of Concentration of Xylose on Fermentation.

No.	Culture No.	0.1 N volatile acid in 100 cc. of culture.			
		2 per cent xylose.	3 per cent xylose.	4 per cent xylose.	5 per cent xylose.
		cc.	*cc.*	*cc.*	*cc.*
1	487–1	74.5	120.6	93.6	93.6
2	41–11	86.2	100.0	127.2	104.7
3	55–9	82.4	77.8	127.4	104.8
4	69–19	90.3	164.8	137.6	109.8
5	69–30	95.0	154.4	115.2	93.6
6	118–5	90.3	138.8	101.8	82.2
7	118–8	95.7	112.8	111.4	72.0

Effect of Reaction.—The behavior of the xylose fermenters in culture media of different reactions was investigated. From previous work it seemed probable that the xylose bacteria would be resistant to relatively large amounts of acid. The correctness

of the assumption can be demonstrated in two ways. The first is by growth in a medium of varying reactions from alkaline to highly acid. Table XVII gives the initial reaction of the culture medium in terms of the hydrogen ion exponent pH and the amount of acid formed. Unlike the earlier experiments, no attempt was made to neutralize the acid formed during fermentation.

The 2 per cent xylose-yeast-water was adjusted to different hydrogen ion concentrations, from pH 5.2 to 8.0. The effect of this variation in the initial hydrogen ion concentration is plainly

TABLE XVII.

Effect of Reaction on the Xylose-Fermenting Bacteria, after 14 Days at 30°C.

No.	Culture No.	1 N sodium hydroxide added.	Reaction at beginning.	0.1 N acid in 100 cc. of culture.	
				Total.	Volatile.
		cc.	*pH*	*cc.*	*cc.*
1	487–1	Control.	5.2	96.4	49.9
2	41–11	"	5.2	122.2	56.7
3	487–1	0.3	5.6	102.6	41.3
4	41–11	0.3	5.6	128.2	71.5
5	487–1	0.5	6.2	106.5	57.5
6	41–11	0.5	6.2	127.8	69.5
7	487–1	0.8	7.4	113.6	64.7
8	41–11	0.8	7.4	132.8	77.5
9	487–1	1.1	8.0	116.2	64.3
10	41–11	1.1	8.0	131.8	
11	487–1	$MgCO_3$	7.2		13.7
12	41–11	$MgCO_3$	7.2		28.5

seen in the yield of acid. The most alkaline solutions gave the highest acid formation, although the difference in the amount of acid produced between pH 7.4 and 8.0 is not great. If the total yield of acid obtained in this experiment is compared with that obtained in previous tests where calcium carbonate was used, it will be noted that only about half as much acid was formed. In other words, these organisms cannot form the maximum amount of acid in the absence of a neutralizing agent; *i.e.*, the acid in amounts greater than 12 per cent of normal acid injures the physiological function of the bacteria.

The value of magnesium as a neutralizing agent for the xylose-fermenting bacteria was included in this test but with unfavorable results. Possibly the magnesium salts are toxic to the proper growth of the xylose organisms.

Rate of Fermentation of Xylose.

It has already been pointed out that the fermentation of xylose takes place rapidly provided desirable conditions prevail. Particular importance is attached to the rate of acid production from xylose, since this fermentation must be rapid if it is to be of

TABLE XVIII.

Rate of Fermentation of Xylose.

Time.	0.1 N acid in 100 cc. of culture.								
	Culture 41-11.			Culture 55-9.			Culture 118-8.		
	Total.	Volatile.	Non-volatile.	Total.	Volatile.	Non-volatile.	Total.	Volatile.	Non-volatile.
days	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
1	54.64	30.40	24.24	58.76	33.80	24.96	57.10	27.80	29.30
2	89.68	49.20	40.48	92.78	49.40	42.38	92.16	48.80	43.36
3	123.72	64.60	59.12	126.80	Lost.	Lost.	126.80	66.60	60.20
4	157.74	86.00	71.74	153.60	82.20	71.40	151.96	83.80	68.16
5	170.52	96.80	73.72	167.02	96.20	70.82	176.70	106.80	69.90
6	194.84	106.40	88.44	190.72	101.80	88.92	195.26	107.60	87.66
7	192.78	104.20	88.58	197.94	107.00	90.94	215.46	120.20	95.26
8	207.22	115.20	92.02	207.64	114.80	92.84	224.74	130.40	94.34
10	222.68	121.20	101.48	221.66	116.96	104.70	258.78	143.78	115.00
12	220.62	Lost.	Lost.	226.82	119.20	107.62	257.74	144.00	113.74
14	230.10	122.00	108.10	221.66	119.40	102.26	260.84	Lost.	Lost.
16	226.82	124.00	102.82	221.66	126.00	95.66	262.90	151.40	111.50

practical value. Additional evidence of the speed of this process in a 3 per cent xylose-yeast medium is shown in the figures of Table XVIII. From the outset of the experiment it was realized that time would not permit exact determinations of the volatile and non-volatile acids as in certain previous tests, therefore only approximate analyses of the acids were made each day. Cultures 41–11, 55–9, and 118–8 were selected for this work. The total acid produced was determined by neutralizing the cultures daily

with N sodium hydroxide, the volatile acid by the phosphate method, and the non-volatile acid by subtraction. The most noteworthy fact brought out by the results of the table is the rapidity of xylose fermentation. Practically all the acid is formed within 8 to 10 days after inoculation under the conditions of this experiment. Perhaps under different conditions, more favorable

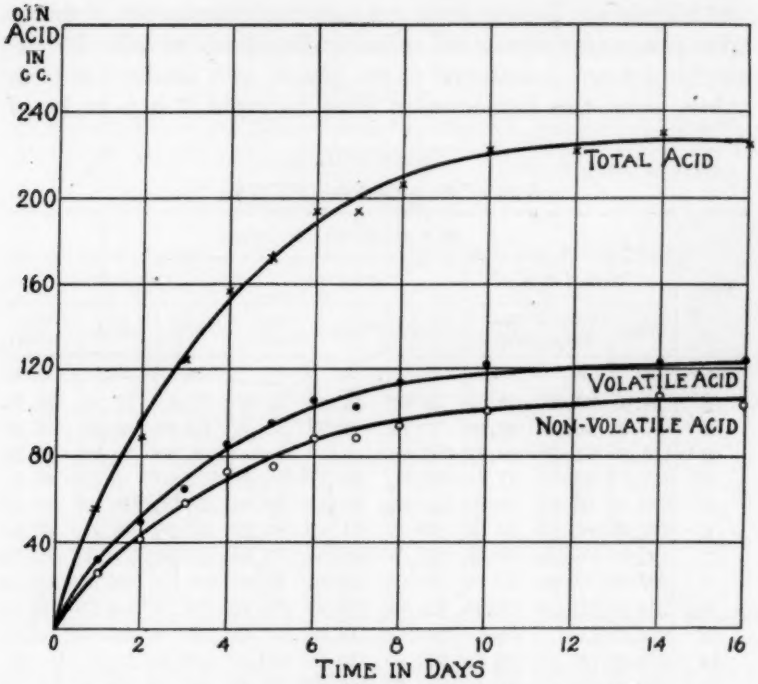

CHART 1. Curves show rate of acid production by Culture 41–11.

temperature, oxygen tension, and reaction, the breaking down of xylose would be even more rapid. An examination of the products of fermentation of the different organisms at varying intervals of time shows that the three organisms exhibit a fairly close agreement. Culture 118–8 perhaps ferments xylose somewhat more rapidly than either of the other cultures. By combining the results obtained over the whole period of the determinations and by taking the mean of the same, an approximate idea

of the ratio of the products formed may be obtained. In the case of Culture 41–11 the mean ratio between the volatile and non-volatile acids calculated as acetic and lactic is 44.2 to 55.7; for Culture 55–9 it is 44.4 to 55.5; and for Culture 118–8 it is 44.8 to 55.1. The proportion of volatile to non-volatile acid in the different cultures is almost identical. This same ratio with some slight fluctuations is maintained throughout the entire fer-

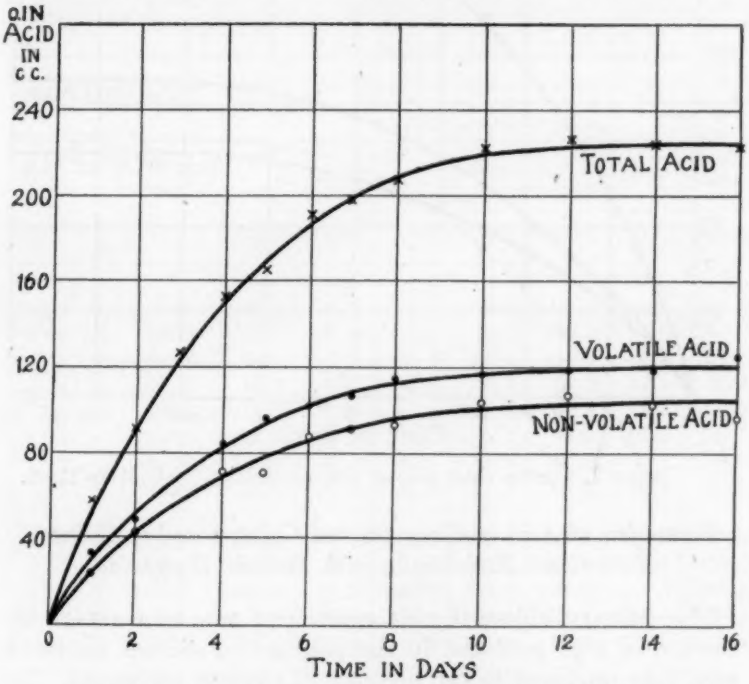

CHART 2. Curves show rate of acid production by Culture 55–9.

mentation. Owing to variations in the individual cultures (a separate culture was used for each analysis) it is difficult to secure an exact comparison of these cultures. Inspection of Charts 1 to 3 will show the general progress of the fermentation, as well as the ratio between the volatile and non-volatile acids. The rise in acid is very rapid at first until there is a concentration equivalent to about 200 cc. of 0.1 N acid per 100 cc., then the process proceeds more slowly. The maximum amount of acid is found about 14 to 16 days after inoculation.

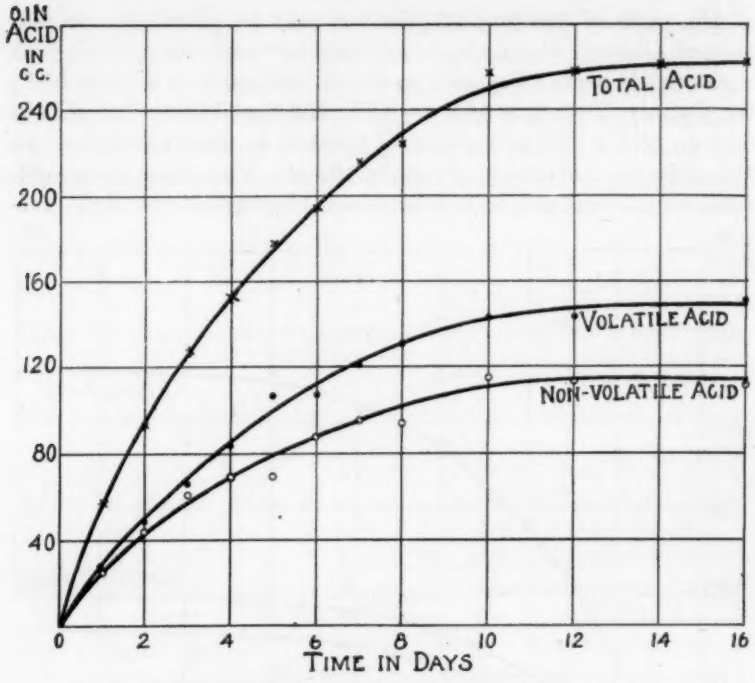

CHART 3. Curves show rate of acid production by Culture 118–8.

Formation of Acid in Unneutralized Cultures and in Cultures Neutralized Periodically with Sodium Hydroxide.

The primary object of this experiment was to compare the amount of acid produced in the presence of sodium hydroxide with that produced in the presence of calcium carbonate. The reaction of the medium at the beginning was pH = 5.6. Each day the cultures containing bromocresol purple were removed from the incubator and sterilized and normal alkali was added until the culture was brought back to the color of the control. At the end of 12 days the cultures ceased to produce acid as shown by the color of the indicator. They were then removed and analyzed. Table XIX presents the results of the analyses.

The figures of the table were obtained from two separate experiments in which different batches of culture media were

used. Nos. 1 to 8 represent one batch of medium and Nos. 9 to 13 another batch of medium. No doubt the higher acid production in the case of Cultures 118–2 to 118–13 is due to the variations in culture media and variations in different batches of xylose. It is of interest to note the relation between the volatile and non-volatile acids. Here, as in former tests, the ratio based on normality was about 50 of volatile to 50 of non-volatile acid.

TABLE XIX.

Comparison of Acid Production in Neutralized and in Unneutralized Cultures.

No.	Culture No.	0.1 N acid in 100 cc. of culture.						
		No alkali added.			Sodium hydroxide added every 2 days.			Calcium carbonate.
		Total.	Volatile.	Non-volatile.	Total.	Volatile.	Non-volatile.	Volatile.
		cc.	cc.	cc.	cc.	cc.	cc.	cc.
1	69–8	104.0	49.8	54.2	179.9	90.4	89.5	110.2
2	69–13	93.0	47.0	46.0	196.5	100.2	96.3	103.0
3	69–19	94.0	47.4	46.6	202.1	104.6	97.5	108.5
4	69–23	93.0	46.2	46.8	162.1	84.2	77.9	99.5
5	69–26	105.0	51.6	53.4	188.7	99.6	89.1	96.0
6	69–27	104.0	50.0	54.0	182.1	90.8	91.3	99.7
7	69–30	100.0	47.6	52.6	188.7	95.6	93.1	94.8
8	69–32	83.0	41.4	41.6	195.3	95.8	99.5	99.7
9	118–2	103.4	48.4	55.0	252.0	124.8	127.2	118.8
10	118–4	101.8	47.6	54.2	255.6	126.0	129.6	122.4
11	118–5	106.0	58.0	48.0	253.2	137.6	115.6	126.4
12	118–8	119.4	63.4	56.0	253.2	137.8	115.4	127.0
13	118–13	75.6	37.8	37.8	142.6	68.8	74.6	114.8

In a 2 per cent xylose-yeast-liquid medium without a neutralizing substance, the production of acid ceased at about 10 per cent N acid per 100 cc. of culture. This acidity, as shown elsewhere, represents about one-half the xylose or approximately one-half the acid formed where basic substances are present.

The cultures to which sodium hydroxide or calcium carbonate was added gave nearly the same yield of volatile acid. It appears that the basic substances, calcium carbonate and sodium hydroxide, are of equal value for this fermentation.

Fermentation of Various Sugars by the Xylose Bacteria.

The value of the fermentation test as an aid in the identification of bacteria is well known. It is frequently used as a means of subdividing types of bacteria which otherwise are difficult to distinguish. In the case of the xylose fermenters two points were noted: (1) gas production, (2) volatile acid production.

For this study, 2 per cent of xylose, glucose, sucrose, lactose, and the related compound, mannitol, were used in yeast-water solution. 24 hour cultures of the organisms were inoculated into

TABLE XX.

Fermentation of Xylose, Glucose, Sucrose, Lactose, and Mannitol by the Xylose Fermenters.

No.	Culture No.	Xylose.		Glucose.		Sucrose.		Lactose.		Mannitol.	
		At beginning.	At end.	At beginning.	At end.	At beginning.	At end.	At beginning.	At end.	At beginning.	At end.
		pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
1	487–1	5.6	3.6	6.0	4.0	6.0	Lost.	5.8	4.4	6.6	4.5
2	41–11	5.6	3.6	6.0	4.0	6.0	3.6	5.8	3.7	6.6	4.3
3	55–9	5.6	3.6	6.0	4.0	6.0	3.5	5.8	4.4	6.6	4.3
4	69–19	5.6	3.5	6.0	3.6	6.0	3.5	5.8	4.4	6.6	4.3
5	69–30	5.6	3.5	6.0	3.6	6.0	4.2	5.8	3.7	6.6	4.3
6	118–5	5.6	3.5	6.0	3.6	6.0	3.6	5.8	4.4	6.6	4.3
7	118–8	5.6	3.5	6.0	3.6	6.0	3.6	5.8	4.4	6.6	4.3

fermentation tubes of media containing the various sugars. The organisms failed to develop gas from any of the sugars. After 2 weeks, the cultures were removed and growth was measured by the change in reaction. Table XX presents a summary of the results obtained.

The hydrogen ion concentration of these cultures was determined colorimetrically and all showed a decided change in reaction. The uninoculated controls gave almost the same hydrogen ion concentration as at the beginning.

Additional data concerning the fermentation of sugars are given in Table XXI. Here, the cultures were incubated for 2 weeks and then analyzed for volatile acid by the phosphate method. The figures of the table represent the amount of volatile

acid minus that of the uninoculated control. The results show that all the substances used are fermented by these organisms. It is true that the total volatile acid formed varies with the different substances; the yield from xylose is from two to three times as great as that from any of the other compounds. Next to xylose, the highest production of volatile acid was found with fructose. The substances glucose, sucrose, lactose, and mannitol are fermented with approximately the same yield of acid. It is of interest to note the variation in acid production among the

TABLE XXI.

Fermentation of Various Sugars and Related Compounds.

No.	Culture No.	0.1 N volatile acid in 100 cc. of culture.					
		Xylose.	Fructose.	Glucose.	Sucrose.	Lactose.	Mannitol.
		cc.	cc.	cc.	cc.	cc.	cc.
1	487–1	114.2	56.0	29.0	11.4	24.0	21.0
2	41–11	122.6	60.0	33.6	35.4	32.0	26.2
3	55–9	122.6	58.8	31.6	37.8	10.6	36.4
4	69–19	120.8	48.0	29.6	21.0	27.2	19.4
5	69–30	120.8	55.2	28.0	22.0	24.4	20.4
6	118–5	130.8	52.0	28.0	22.0	19.4	21.2
7	118–8	125.6	54.4	24.4	23.4	6.0*	16.4

* Contamination.

different cultures. Apparently these strains of the xylose fermenters show decided differences in amount of volatile acid formed from various sugars. Qualitative tests show the formation of acetic and lactic acid from these compounds and in the case of fructose, the production of mannitol.

Nitrogen Nutrition of the Xylose Fermenters.

Experiments were made by inoculating the xylose bacteria into culture solutions containing different sources of nitrogen and 2 per cent of xylose. Growth was determined by visible changes in the medium to which bromocresol purple was added. In all tests 50 cc. portions of the culture medium (reaction pH = 6.5 to 6.8) in small flasks were used. Table XXII gives the substances employed and their approximate value for the xylose fermenters.

Peptone, beef extract, and gelatin gave evidence of supporting growth, while the other nitrogenous compounds did not support the growth of these organisms. When compared with yeast-water, which contained approximately 0.05 per cent of nitrogen, it is clear that the compounds of nitrogen or the concentration employed in this experiment are not nearly so favorable for growth.

TABLE XXII.

Growth of Xylose Bacteria on Different Nitrogenous Compounds.

No.	Culture No.	Beef extract 0.3 per cent.	Peptone 0.3 per cent.	Gelatin 0.3 per cent.	Asparagine 0.2 per cent.	Urea 0.1 per cent.	Ammonium sulfate 0.2 per cent.
1	487–1	+	+	+	–	–	–
2	41–11	+	+	+	–	–	–
3	55–9	+	+	+	–	–	–
4	69–19	+	+	+	–	–	–
5	69–30	+	+	+	–	–	–
6	118–5	+	+	+	–	–	–
7	118–8	+	+	+	–	–	–

+ = Growth. – = No growth.

SUMMARY.

Xylose is readily fermented by bacteria which are found in fresh silage, sauerkraut, and manure, and also in certain soils. All attempts to break down xylose by means of the microorganisms commonly studied in the laboratory failed. The following yeasts, *Carlsberg* I, *Burgundy*, the lactose-fermenting yeasts, *amara* and *Hansen*, as well as the bacteria, *Bacillus mycoides*, *Bacillus subtilis*, *Bacillus azotobacter*, and a high acid-producing form from corn silage, did not show any appreciable growth in a medium with xylose as the only carbohydrate.

The xylose fermenters are easily isolated in pure culture by inoculating xylose-yeast-liquid medium with silage, sauerkraut, etc., and subsequent plating on xylose-yeast-agar from these liquid cultures; or they may be isolated directly from the sources named on xylose-yeast-agar plates. On solid culture media these organisms grow scantily, producing small dot-like colonies about 1 mm. in diameter. Longer periods of incubation do not cause any noticeable increase in the size of the colonies.

The fermentation of xylose takes place either in the presence of free oxygen or in a limited supply. These organisms will grow throughout the whole length of an agar stab. They are facultative anaerobes growing best with a lowered oxygen tension. In relation to reaction, the xylose-fermenting bacteria grow within a relatively wide range, in xylose-yeast-water medium from a hydrogen ion concentration of about pH = 3.0 to 8.6.

The main products formed in the fermentation of xylose are acetic acid and lactic acid. From determinations of the water of crystallization it was found that the lactic acid produced by the fermentation of xylose belongs to the racemic type. These two acids represent about 90 per cent of the sugar consumed. The relation of acetic acid to lactic acid is approximately 43 gm. of the former to 57 gm. of the latter, and approaches the theoretical ratio of 40 to 60 that would obtain if these two compounds were the only products resulting from the cleavage of the xylose molecule. The only minor products found were small amounts of carbon dioxide and a trace of ethyl alcohol. Undoubtedly a small amount of the xylose is consumed in the building up of the cells of the bacteria.

The fermentation of the xylose takes place rapidly and within 10 to 12 days after inoculation practically all this sugar is consumed. Periodic determinations of the total and volatile acids formed from xylose show that more than half the acids are produced from the 1st to the 4th day. Hereafter the acid production proceeds more slowly. The proportion of the volatile acid to the non-volatile acid is approximately the same throughout the entire period of fermentation; namely, 40 of acetic to 60 of lactic acid. The maximum amount of acid per gm. of xylose fermented is obtained in cultures containing from 2 to 3 per cent of xylose.

Sugars other than xylose, such as glucose, sucrose, lactose, and fructose, as well as the related compound mannitol, are fermented by these organisms with the production of volatile and non-volatile acids; however, the total amount of acids formed is small as compared with that from xylose. From qualitative tests, it appears that here again acetic and lactic acids are formed but in what proportion is not known. Fructose is fermented rapidly with the production of acids and of mannitol. This phase of the work is receiving further study.

In yeast-water or cabbage juice solutions these organisms ferment xylose much more rapidly than in water solutions containing nitrogen from beef extract, peptone, or gelatin.

The authors wish to call attention to the occurrence of these organisms in silage and their possible significance in the production of the acids of silage.

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EXPLANATION OF PLATE 5.

FIG. 1. Xylose fermenter. Culture 41–11. A 24 hour culture on xylose-yeast-agar. Stained with fuchsin. × 1,200.

FIG. 2. Xylose fermenter. Culture 55–9. A 24 hour culture on xylose-yeast-agar. Stained with fuchsin. × 1,200.

FIG. 3. Xylose fermenter. Culture 118–8. A 24 hour culture on xylose-yeast-agar. Stained with fuchsin. × 1,200.

FIG. 1.

FIG. 2.

FIG. 3.

(Fred, Peterson, and Davenport: Acid fermentation of xylose.)

ACTION OF SALTS UPON THE METABOLISM OF NERVES.

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(Received for publication, July 7, 1919.)

INTRODUCTION.

The question of the relation of functional to metabolic activity in the nerve fiber has been made the subject of numerous investigations by Tashiro and his students. In the course of these investigations the following facts have been brought out.

1. Living nerve fibers give off carbon dioxide at rates which vary for various animals and for various nerves of the same individual (1).

2. The metabolic rate is not the same in all portions of a single nerve: Thus it has been shown that the rate of metabolism of a sensory nerve is highest in that portion of the nerve nearest to its peripheral ending and least near its central termination and that the reverse is true of a motor nerve (2, 3, 4).

3. Electrical stimulation of a nerve by means of a weak tetanizing current increases its rate of metabolism to about two and one-half times that of a resting nerve (1).

4. If a nerve is allowed to respire in an atmosphere of pure hydrogen the carbon dioxide production from it is found to be decidedly less than that of a similar nerve which has respired in an atmosphere containing oxygen. Further, electrical stimulation of a nerve in an atmosphere of hydrogen fails to bring about a marked increase of carbon dioxide production (5).

5. A 10° increase of temperature increases the rate of carbon dioxide production approximately 100 per cent (1, 6).

6. The treatment of a nerve with small concentrations of the narcotic drugs ethyl urethane and chloral hydrate brings about a state of increased irritability and corresponding with it a condition of increased rate of carbon dioxide production. A treatment with greater concentrations of these same drugs or with small concentrations for a longer period of time brings about a condition of depressed irritability or narcosis and with it a decreased rate of carbon dioxide production (7).

7. There is evidence for the general statement that nerves which conduct an impulse rapidly have a higher rate of metabolism than those which conduct at a lesser rate (3).

The interpretation which has been built up from these and similar facts is that the functional activity of the nerve is fundamentally a dynamic, metabolic process. If this interpretation is correct, we may then predict that any physical or chemical treatment which serves to throw a nerve into functional activity will be found also to increase its metabolic activity and that we may measure this, as was done in the experiments quoted above, by the rate of carbon dioxide production.

The action of inorganic salts upon physiological conditions or states constitutes one of the frequently studied subjects of physiology. Most of the experiments which have been devised in an effort to throw light upon the mechanism of salt action fall into one of the three following classes. First, experiments in which the living organisms or tissues are treated with solutions of various salts and observations are made upon the physiological effect of such treatment. Second, the effect of salts upon various processes and conditions such as speed of oxidation, surface tension, etc., has been studied *in vitro* and the results of such studies have been carried over to explain, by analogy, the physiological action of salts. The results of such studies are of a very considerable value, but there is always very grave danger that the conditions of the experiments *in vitro* are far from being represented in the living tissues. Third, a method of study has less frequently been undertaken in which the action of salts upon the functional condition of tissues has been studied simultaneously with some other process occurring within the tissues. This last is the type of study which has here been carried out and which is the basis of this report. The ideal material for such studies appeared to be the nerve on account of the fact that in such material the complicating factors of movement, cell division, secretion, etc., are absent, the only functional changes known to occur in the nerve being electrical and chemical changes.

The action of salts upon nerves has been carefully studied by Mathews (8) who has shown that when nerves are treated with salts a characteristic action (stimulation or depression) occurs, to a degree and within a period of time which is characteristic for each salt.

It was suggested to me by Professor Mathews that I undertake, under Professor Tashiro's direction, a study of the action of certain stimulating and depressing salts on the carbon dioxide production in nerve, in order to correlate their effect on metabolism and irritability.

I am much indebted to Professor Mathews for thus suggesting this problem.

EXPERIMENTAL.

Method.

The effect of salts upon the metabolism of nerves was determined by comparing the rate of carbon dioxide production of a nerve treated with a given salt solution, with that of a control nerve kept in Ringer's solution, such comparisons being made in Tashiro's biometer (9). Quantitative determinations were also made upon the carbon dioxide production of nerves under various conditions by means of his Apparatus III (10).

No doubt the ideal way of determining the effect of a salt upon the metabolism of a nerve would be a device for measuring the carbon dioxide produced during the time that the nerve is being bathed in a given salt solution, but on account of the fact that the presence of even a minute quantity of an aqueous solution in the chamber of the apparatus introduces a serious error into the determination of carbon dioxide, the following procedure was adopted.

A frog was killed by decapitation, a nerve muscle preparation quickly made, and hung in a moist chamber with the nerve dipping into the salt solution to be studied and the muscle attached to a lever writing on a slowly revolving drum. Stimulation of the nerve by the salt solution is then indicated by the twitching of the attached muscle. To study the carbon dioxide production of a nerve so treated it was cut free from the muscle and after the removal of all adherent solution by means of filter paper it was placed in the respiration chamber of the carbon dioxide apparatus.

Of course the physiological condition of a nerve at the time of its removal from the salt solution gives us very little evidence as to its physiological state during the time that it remains in the

respiration chamber, so that in order to determine this point controls were run in which the condition of the removal of the nerve from the salt solution to the respiratory chamber were duplicated as nearly as possible, except that the nerve was not cut free from the muscle. This was accomplished by lifting the nerve carefully from the salt solution, removing adherent solution by means of filter paper, and allowing it to remain in the moist chamber on a clean glass cover-slip. The results of these control experiments as well as the results of the determinations of carbon dioxide are given under the discussion of each salt.

Sodium Chloride.—When the sciatic nerve of a frog is treated in the manner described above with an $\frac{}{8}$ solution of sodium chloride, which is isotonic with frog blood, there is no obvious effect for a latent period of about 120 minutes. At about the end of this latent period a few twitchings indicate that the nerve is being stimulated by the salt solution. These twitchings are rhythmical and become gradually more vigorous and may persist for as long as several hours (7). If the nerve is removed to a glass plate within the moist chamber at any time during the latent period there is no indication of any stimulation of the nerve, but if it is removed from the solution after the stimulation has begun there is a period of about 2 or 3 minutes in which all twitchings stop, except those which are due to the handling of the nerve. Following this second short latent period there occurs a short period of vigorous stimulation which persists for from 1 to 4 minutes.

In the series of experiments which are summarized in Table I the carbon dioxide production from nerves kept in the sodium chloride solution for various periods of time was compared with that of control nerves kept in Ringer's solution for similar periods of time. In these experiments equal weights of the two sciatic nerves were placed in the sodium chloride and Ringer's solutions and at the end of the various periods of time indicated in the table their relative rates of carbon dioxide production were determined in the biometer. For the periods of time up to 135 minutes there was no detectable difference in the rates of carbon dioxide production from the two nerves, but for longer periods of time there was noted a slight but certain difference in favor of the nerve kept in the solution of sodium chloride. These ex-

TABLE I.

Comparison of CO_2 Production of Two Nerves, One Kept in $\frac{N}{8}$ NaCl, the Other in Ringer's Solution.

Nerve A. Time in $\frac{N}{8}$ NaCl.	Nerve B. Time in Ringer's.	Number of experiments.	Result.
min.	*min.*		
1	1	1	A = B
2	2	2	A = B
2	2	2	A > B
2	2	1	A < B
5	5	4	A = B
5	5	1	A < B
10	10	5	A = B
10	10	3	A > B
10	10	3	A < B
15	15	5	A = B
15	15	1	A > B
20	20	4	A = B
24	24	1	A > B
25	25	3	A = B
25	24	1	A = B
30	30	3	A = B
30	30	1	A > B
40	40	3	A = B
40	40	1	A > B
40	40	1	A < B
44	44	1	A = B
55	55	2	A = B
60	60	1	A = B
70	70	2	A = B
80	80	1	A = B
90	90	1	A = B
100	100	1	A = B
114	115	1	A = B
135	135	1	A = B
135	135	2	A > B
139	139	1	A = B
141	141	3	A > B
150	150	1	A > B
160	160	2	A > B
180	180	2	A > B

experiments indicate that there is an increased production of carbon dioxide from a nerve treated with sodium chloride, in isotonic solution, and that this period of increased metabolic activity comes on at a time which corresponds to the period of stimulation by the salt. In order to test further the actual constant occurrence of this difference as well as its relation to the period of stimulation, a second series of experiments was tried in which a nerve muscle preparation was suspended in a moist chamber and the nerve was allowed to dip into the solution of sodium chloride until the period of stimulation, as evidenced by the vigorous contractions of the muscle, was well developed. During this period of stimulation, the stimulated and the control nerve were removed from the solutions to the biometer and their rates of carbon dioxide production compared (Table II). In this series of experiments more time elapsed between the removal of the nerves from the solutions and the determination of their carbon dioxide production than in the first series, owing to the necessity of bringing the nerves to equal weight.

TABLE II.

*Comparison of CO_2 Production from a Nerve (B) Stimulated by an $\frac{N}{8}$ Solution of NaCl with That of a Control Nerve (A) in Ringer's Solution.**

Number of experiments.	Result.
6	A > B
2	A < B
1	A < B(?)
6	A = B

* Time not recorded.

This series of experiments is rather less convincing than the first, for in none of them was there more than a slight difference in the rate of carbon dioxide production noted and out of fifteen experiments only six confirmed the conclusion suggested by the first series. However, in view of the short duration of the period of stimulation following the removal of the nerve from the salt solution, it appears that the reason for this discrepancy in results might be that a greater length of time is required in the second series for the transfer of the nerves from the solution to the

biometer. In order to test this possibility a third series of experiments was carried out in which a nerve was kept in a solution of sodium chloride until it was being vigorously stimulated by the salt solution. It was then removed from the solution to the biometer, a control nerve kept in Ringer's solution being placed in the other. The nerves were weighed after the experiment was complete and the results recorded only for those experiments in which there was a difference of but a few mg. in the weight of

TABLE III.

*CO_2 Production of a Nerve Stimulated by $\frac{N}{8}$ NaCl Compared with That of a Control Nerve.**

Nerve A, being stimulated by $\frac{N}{8}$ NaCl; Nerve B, kept in Ringer's solution.

Weight of Nerve A.	Weight of Nerve B.	Number of experiments.	Result.
mg.	*mg.*		
24	26	1	A > B
26	25	1	A > B
23	28	1	A = B
17	16	1	A > B
20	21	1	A > B
22	21	1	A > B
30	29	1	A > B
25	25	1	A > B
24	22	1	A = B
19	18	1	A = B
(?)†	(?)†	4	A > B

* The nerves were weighed after the CO_2 production had been compared.

† The nerves in these experiments were of the same weight but the weight was not recorded.

the nerves (Table III). In this series of fourteen experiments eleven show a greater production of carbon dioxide by the nerve stimulated by means of the sodium chloride solution. This series of experiments taken together with those of the first series (Table I) indicate clearly that a period of increased carbon dioxide production occurs in a nerve coincident with a period of stimulation by an isotonic solution of sodium chloride.

No quantitative determinations were made on the carbon dioxide production by nerves treated with sodium chloride

solution, on account of the fact that the period of stimulation following the removal of the nerve from the salt solution persists for so short a time, certainly less than that required for respiration in the chamber of the quantitative apparatus.

Although quantitative determinations were not made, there can be no doubt there is an increase in the rate of carbon dioxide production from a nerve treated with an isotonic solution of sodium chloride for periods of about 130 or 150 minutes. This period of increased metabolic rate corresponds to a short period of increased functional activity of the nerve fiber which follows the removal of the nerve from the salt solution.

Sodium Sulfate.—While the experiments with sodium chloride indicate that the metabolic activity of a nerve is increased during salt stimulation, we are given no clue as to the extent of such increase. It is of interest to know if this increase is of the same order as that due to electrical stimulation. It therefore appeared to be of interest to select a salt which would not only stimulate a nerve, but one in which the period of stimulation would continue for a sufficiently great length of time after the removal of the nerve from the solution to enable quantitative determinations to be made during this period of stimulation. After the trial of several salts sodium sulfate was found to satisfy these conditions.

When a nerve muscle preparation is hung in a moist chamber with the nerve dipping into an isotonic ($\frac{N}{8}$) solution of sodium sulfate, a series of events occurs which is very similar to that occurring in the case of sodium chloride, except that the latent period is only 20 minutes in length and the contractions which follow the latent period are stronger and so rapid as to blend into a long low tetanus. When a nerve is removed from the solution and placed on a glass plate within the moist chamber, at any time during the latent period, no contractions occur (except in a few cases which will be noted later). If, however, the nerve is similarly removed from the solution during the period of salt stimulation, there follows after a second latent period of 2 or 3 minutes, a high tetanus which persists for about 35 or 45 minutes (Fig. 1).

A series of experiments was carried out in which the two sciatic nerves of a frog were removed as quickly as possible and placed, the one in an $\frac{N}{8}$ solution of sodium chloride and the other in an $\frac{N}{8}$ solution of sodium sulfate. At the end of various periods

FIG. 1. 1. Latent period. Nerve bathed by $\frac{N}{8}$ Na_2SO_4.
2. Nerve removed from stimulating solution.
2 to 3. Latent period.
3. Beginning of second stimulation; *i.e.*, stimulation after removal from the $\frac{N}{8}$ Na_2SO_4.

TABLE IV.

Comparison of CO_2 Production of Two Nerves, One Kept in $\frac{N}{8}$ NaCl, the Other in $\frac{N}{8}$ Na_2SO_4.

Nerve A. Time in $\frac{N}{8}$ NaCl.	Nerve B. Time in $\frac{N}{8}$ Na_2SO_4.	Number of experiments.	Results.
min.	*min.*		
1	1	2	A = B
1	1	1	A < B
2	2.5	1	A = B
5	5	2	A = B
4	4	1	A = B
8	8	1	A = B
10	10	4	A = B
15	15	2	A = B
20	20	3	A = B
20	20	1	A < B
25	25	1	A = B
25	25	3	A < B
30	30	1	A < B
30	30	1	A = B
40	40	2	A < B
45	45	1	A = B
55	55	1	A = B
55	55	2	A < B
60	60	2	A = B
60	60	1	A < B
70	70	1	A > B
70	70	1	A < B
80	80	2	A = B
95	95	1	A = B
95	95	1	A < B
100	100	2	A = B
110	110	1	A > B
114	114	1	A = B
120	120	1	A = B
120	120	1	A > B
140	140	2	A > B
180	180	1	A > B
240	240	1	A = B
300	300	1	A = B

of time these nerves were removed from the solutions and their carbon dioxide production was compared in the biometer (Table IV).

For periods of time up to about 25 minutes there is no constant difference in the rate of carbon dioxide production from the two nerves, but from this time up to about 70 minutes there is a greater production of carbon dioxide from the nerve kept in the solution of sodium sulfate. There is again no marked difference noted until the periods from 120 to 180 minutes, when there is a greater production of carbon dioxide from the nerve kept in the solution

TABLE V.

CO_2 Production of a Nerve Stimulated by $\frac{N}{8}$ Na_2SO_4 Compared with That of a Control Nerve in $\frac{N}{8}$ NaCl or in Ringer's Solution.

Number of experiments.		Result.
Nerve A kept in $\frac{N}{8}$ NaCl.	Nerve B stimulated by $\frac{N}{8}$ Na_2SO_4.	
8	8	A < B
1	1	A = B
1	1	A = B(?)

Weight of Nerve A kept in Ringer's.	Weight of Nerve B stimulated by $\frac{N}{8}$ Na_2SO_4.	Number of experiments.	Result.
mg.	*mg.*		
30	30	1	A < B
25	23	1	A < B
24	26	1	A = B
24	25	1	A < B
31	29	1	A < B

of sodium chloride. These periods of increased metabolism have a general correspondence to the periods of stimulation by the salts used in these experiments.

In order to study further the relation between the period of increased metabolic activity and the period of stimulation by sodium sulfate, a second series of experiments was carried out in which a nerve muscle preparation was set up in the moist chamber with the nerve dipping into $\frac{N}{8}$ sodium sulfate. The nerve was removed and its carbon dioxide production compared with that

of a control nerve kept in Ringer's solution, after the period of stimulation was well developed (Table V).

In twelve out of fifteen of these experiments the carbon dioxide was found to be greater from the nerve which was being stimulated by the sodium sulfate than from the control nerve which had been kept in $\frac{N}{8}$ sodium chloride solution or in Ringer's solution. The results are the same in these experiments when the nerves are weighed before the experiment as when they are weighed after the experiment is completed. These experiments show that the period of increased metabolic activity is also the period of stimulation by the salt.

The fact that the period of stimulation after the removal of the nerve from the salt solution persists for so long a time (35 to 45 minutes) makes it possible to make quantitative determinations of the production of carbon dioxide during the time that this period of stimulation still exists in the nerve. In order to determine the degree to which the production of carbon dioxide is increased by stimulation with sodium sulfate, a nerve muscle preparation was set up in the moist chamber and when the contractions of the muscle indicated that the nerve was being stimulated, it was removed to the respiration chamber of the quantitative apparatus.[1] The results of these determinations are given in Nos. 7, 8, 9, and 10 of Table VI.

These experiments show that the carbon dioxide production of a nerve stimulated by sodium sulfate is increased from 2.5×10^{-7} to 5.5×10^{-7} gm. of carbon dioxide per centigram of tissue for each 10 minutes of respiration; *i.e.*, the rate of metabolism of the nerve is increased 2.2 times by the salt stimulation. This increase, while it is less than that noted when a nerve is stimulated electrically (found by Tashiro (1) to increase the metabolism of a nerve 2.4 times) is of the same order of increase. That sodium sulfate stimulation is less intense than electrical is indicated by the fact that if an electrical stimulus just strong enough to be felt on the tongue is sent into the nerve at the height of the

[1] The respiration period used in these experiments was 30 minutes and as the nerves were kept in the solution from 20 to 30 minutes, the nerve had been out of the body of the animal for about 1 hour before the experiment was complete. This fact should be kept in mind when comparing these results with the quantitative results of Tashiro.

contractions due to the salt stimulation, the muscle responds with still higher contractions. In other words sodium sulfate stimulation is not maximal. This lesser grade of stimulation may be able to account for the fact that the metabolism of a nerve is not increased so much by sodium sulfate as by electrical stimulation.

The question as to the metabolic activity of a nerve during the latent period of sodium sulfate action appeared to be of special interest on account of the fact that it was frequently noted that

TABLE VI.

*Effect of $\frac{N}{8}$ Na_2SO_4 on the Sciatic Nerve of the Frog.**

No.	Solution.	Time in solution.	Condition of nerve.	CO_2 produced by 10 mg. of nerve in 10 min.	Range of temperature.
		min.		*gm.*	*°C.*
1	$\frac{N}{8}$ Na_2SO_4	5	Irritable.	4.1×10^{-7}	18–21
2	Ringer's.	5	"	4.3×10^{-7}	18–22
3	$\frac{N}{8}$ Na_2SO_4	10	"	4.1×10^{-7}	17–20
4	Ringer's.	10	"	3.8×10^{-7}	18–21
5	$\frac{N}{8}$ Na_2SO_4	15	"	3.0×10^{-7}	17.5–20
6	Ringer's.	15	"	2.8×10^{-7}	17.5–22
7	$\frac{N}{8}$ Na_2SO_4	20	Stimulated.	5.5×10^{-7}	17–20.5
8	Ringer's.	20	Irritable.	2.5×10^{-7}	19–21
9	$\frac{N}{8}$ Na_2SO_4	30	Stimulated.	5.4×10^{-7}	18–21
10	Ringer's.	30	Irritable.	2.4×10^{-7}	18–22

* Each entry in this table is the average of several determinations. All determinations were made at temperatures including the extremes given in Column 6 of this table.

a nerve was extremely irritable during and especially near the end of the latent period. This heightened irritability was at times so great that the slamming of a door would cause a contraction of the muscle. In an effort to throw light upon this question, the carbon dioxide production of nerves during various portions of the latent period was studied quantitatively and the results of these determinations compared with the results of studies similarly made upon control nerves kept in Ringer's solution. Several complicating factors have, however, rendered these results unsatisfactory. In the first place nerves do not always show this increased irritability in the latent period, secondly it

does not appear that this state of heightened irritability always remains after the nerve has been removed from the salt solution, and finally some exceptions were noted to the general rule that a nerve removed from the solution during the latent period does not show signs of stimulation. The results of these experiments are given in Table VI. It has been as yet impossible to control the above complicating factors sufficiently well to warrant any conclusion other than, that for periods of time up to 15 min-

TABLE VII.

Comparison of CO_2 Production of Two Nerves, One Kept in $\frac{N}{8}$ KCl, the Other in $\frac{N}{8}$ NaCl or in Ringer's Solution.

Time of Nerve A in $\frac{N}{8}$ NaCl.	Time of Nerve B in $\frac{N}{8}$ KCl.	Number of experiments.	Result.
min.	*min.*		
5	5	2	A > B
10	10	1	A > B
15	15	1	A > B
22	22	1	A > B
30	30	1	A > B
40	40	2	A > B
In Ringer's.			
10	10	2	A > B
15	15	2	A > B
20	20	1	A > B
30	30	1	A > B
40	40	1	A > B
	Stimulated electrically.		
15	15	3	A > B
20	20	1	A > B
30	30	3	A > B

utes, a nerve in an isotonic solution of sodium sulfate has a rate of respiration not markedly different than that of a control nerve kept in Ringer's solution.

Potassium Chloride.—When a nerve is treated with an isotonic solution ($\frac{N}{8}$) of potassium chloride its irritability is speedily abolished so that it does not even respond to the strong stimulus of cutting. This non-irritable or narcotized state persists after the nerve has been removed from the solution but its normal condi-

tion returns when it is placed in sodium chloride or in Ringer's solution. That this depression of irritability is accompanied by a decrease of metabolic activity is shown by a series of experiments in which the carbon dioxide produced by a nerve so treated is compared with that of a nerve of the same animal kept for the same length of time in an $\frac{N}{8}$ solution of sodium chloride or in Ringer's solution. Electrical stimulation of a nerve which has been rendered non-irritable by means of potassium chloride fails to increase markedly its rate of carbon dioxide production (Table VII).

There is no primary increase in the rate of carbon dioxide production, from a nerve treated with a solution of potassium chloride, such as was observed by Tashiro and Adams (7) in the

TABLE VIII.

*Effect of $\frac{N}{8}$ KCl on the Sciatic Nerve of the Frog.**

No.	Solution.	Time in solution.	Condition of nerve.	CO_2 produced by 10 mg. of nerve in 10 min.	Range of temperature.
		min.		*gm.*	*°C.*
1	$\frac{N}{8}$ KCl	5	Non-irritable.	1.9×10^{-7}	20–26
2	Ringer's.	5	Irritable.	4.3×10^{-7}	18–22
3	$\frac{N}{8}$ KCl	30	Non-irritable.	1.59×10^{-7}	20–24
4	Ringer's.	30	Irritable.	2.4×10^{-7}	18–22

* Each entry in this table is the average of several determinations. All determinations were made at temperatures including the extremes given in Column 6 of this table.

case of narcosis by drugs. This fact is interesting in view of the fact that Mathews failed to discover any primary stimulation of a nerve by an isotonic solution of potassium chloride.

The degree to which the metabolism of a nerve is depressed by an $\frac{N}{8}$ solution of potassium chloride has been studied quantitatively and the results are given in Table VIII.

These experiments show that a nerve treated with an $\frac{N}{8}$ solution of potassium chloride has its rate of carbon dioxide production decreased from 4.3×10^{-7} to 1.9×10^{-7} gm. of carbon dioxide per centigram of tissue for each 10 minutes respiration and that a treatment for 30 minutes decreases its rate of carbon

dioxide production from 2.4×10^{-7} to 1.5×10^{-7}. In other words a nerve kept in a solution of potassium chloride for 30 minutes has a rate of metabolism only 0.66 that of a normal nerve kept for the same length of time in Ringer's solution, and the carbon dioxide production of a nerve kept in the potassium chloride solution for 5 minutes is 0.44 that of a nerve kept in Ringer's solution for the same length of time. These results are of the same order as the results of Tashiro and Adams (7) who found that the carbon dioxide production from the claw nerve of the crab, *Labinia canaliculata*, which they rendered non-irritable by means of drugs, was reduced to from 0.53 to 0.35 that of the normal nerve.

SUMMARY.

The facts brought out in the preceding quantitative studies may be easily summarized in the curves of Fig. 2 in which the

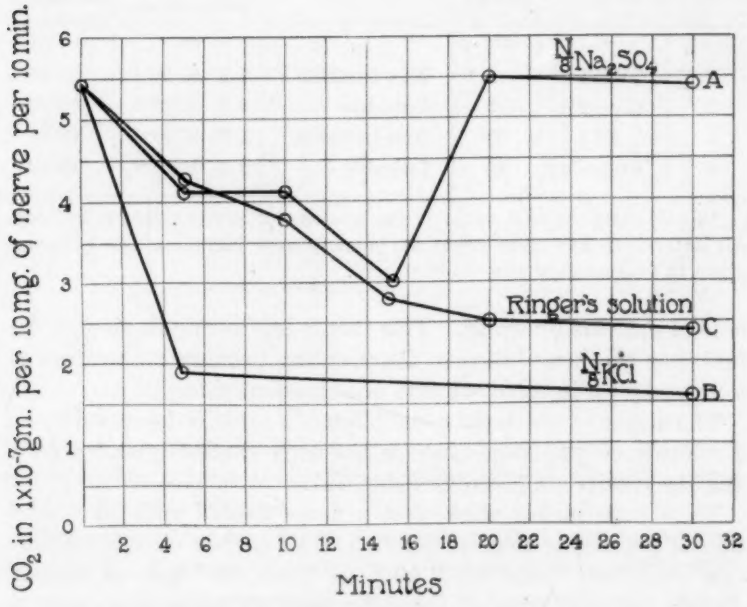

FIG. 2. Quantitative comparisons of CO_2 productions from the nerves treated by (*A*) $\frac{N}{8}$ Na_2SO_4, (*B*) $\frac{N}{8}$ KCl, and (*C*) Ringer's solution.

carbon dioxide produced, expressed in gm. of carbon dioxide per cc. of tissue for each 10 minutes respiration, is represented by the ordinates and the time that the nerves are allowed to remain in the solution by the abscissæ.

The carbon dioxide production of a normal nerve suffers a gradual decrease in rate after the nerve is removed from the body and allowed to remain in Ringer's solution while that of a nerve kept in a solution of sodium sulfate isotonic with frog's blood suffers a similar decrease up to the time that it is stimulated by the salt solution, at which time a sudden increase in the rate of carbon dioxide production occurs. This increase in the rate of metabolism is to the extent of 2.2 times that of the resting nerve.

The increase in the metabolic rate of a nerve by sodium sulfate stimulation is of the order of that of electrical stimulation and indicates that the mechanism involved is the same in both cases and is an additional indication that the relation between stimulation and increased rate of metabolic activity is a causal one or at least that an increased rate of carbon dioxide production is an invariable accompaniment of increased functional activity.

Additional evidence is found in these results against the view of Bayliss (11), who holds that the increased production of carbon dioxide observed when a nerve is stimulated by means of an electrical current may be due not to increased metabolic activity of the nerve fiber but rather to the heating of the nerve by the electrical current. In these experiments there is no obvious means of heating the tissue, or at least there appears to be no greater possibility of heating a nerve by means of an isotonic solution of sodium sulfate than by a similar solution of potassium chloride or by Ringer's solution.

The action of potassium chloride in abolishing the irritability of a nerve and at the same time reducing its metabolic rate is in accord with the broad general thesis of the relations between functional and metabolic activities. The effect of potassium chloride upon the metabolism of nervous tissue rendered non-irritable by its action is similar to that of the drugs studied by Tashiro and Adams and lends further support to the view developed chiefly from the findings of Mathews, Loevenhart, and Verworn that the primary effect of the narcotic agents, drugs, or

salts is upon the chemical, metabolic activity of the living substance.

The suggestion that biological "salt action" in general is upon the chemical activity of the living substrate is supported by this paper.

To Dr. Shiro Tashiro, under whose direction these experiments have been carried out, I wish to express my sincere gratitude.

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SOME DATA CONCERNING THE ALLEGED RELATION OF CATALASE TO ANIMAL OXIDATIONS.

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(Received for publication, July 29, 1919.)

During the past two years W. E. Burge and his collaborators have communicated numerous papers dealing with the enzyme catalase. As a result of the parallelism believed to have been observed between the intensity of oxidation in the animal body and the catalase content, they have concluded that catalase is the enzyme in the body principally responsible for oxidations.

In none of Burge's papers is there any direct evidence to substantiate his hypothesis. The part which catalase plays in the bodily functions is still obscure. Until it has been shown to be capable of inducing some, at least, of the changes which food or tissue undergoes in the oxidative catabolism the discovery that the quantity in which it exists in various tissues runs parallel with the oxidative activity of those tissues is not evidence that it takes part in those oxidations. While the observation of a strict parallelism between intensity of oxidation and catalase content would be interesting in so far as it might give impetus to the performance of more direct experiments regarding the rôle of catalase in biological oxidations, it is not true that such a relation has been sufficiently proved to warrant the formulation of a new theory of oxidation. Indeed there is evidence that Burge's hypothesis is based upon an incomplete analogy; and not only is the analogy incomplete but there are contradictions to it. The last part of this paper is concerned with a few of them.

The following is a concise statement of some of the experiments of Burge and his collaborators.

They have determined the catalase content of heart, leg, and back muscles of several species of animals (1) and found it to diminish in the order given. Regarding the heart muscle as the most active of the three and the muscles of the head as the least active it is argued that the greater catalase content is the cause of the greater activity and by implication of a greater oxidative capacity.

One of Burge's papers (2) is devoted to showing that the catalase content of the muscles of different species of animals varies inversely with the size. The ratio of goose, pigeon, and sparrow catalase found was 46: 104: 121. From these data Burge was led to conclude that the reason for the fact that the metabolism of small animals is greater than that of large animals (when calculated per kilo of body weight) is that the catalase content of the former is greater than that of the latter. The data, however, are inadequate; they represent averages of but few determinations in each case and in this connection it is worth knowing that the fluctuations in the blood catalase of animals of the same species and not widely differing weights exceed 100 per cent. This fact alone does much to detract from the importance of catalase as an oxidizing enzyme.

Phosphorus poisoning (3) is reported to cause a decrease in the liver, heart, and blood catalase. In the case of the last named the data actually show an increased catalase content in two out of five cases. As far as metabolism in phosphorus poisoning is concerned Lusk (4) has shown it to be slightly increased.

Starvation (5) and restricted activity (1) are reported to decrease the catalase content of muscle.

Thyroid feeding (6) was found to decrease the catalase content of the heart and increase that of the blood, and anesthetics (7, 8) were found to decrease the catalase content of blood, but Becht (9) has been unable to duplicate Burge's results in these instances.

Strong emotions (10) aroused in cats by subjecting them to the barking of dogs caused a rise in the catalase content of washed liver tissue. Alcohol (11), saccharin (12), acetone bodies (13), amino-acids (14, 15), and a great variety of food materials (16, 17) produced increases in the catalase content of blood. Pancreatectomy (18), and shock caused by manipulation of the intestines and by bleeding (19) caused a decrease in catalase and another group of foodstuffs (16, 17) produced no change.

In passing, attention may be called to several of Burge's statements which do not harmonize with current knowledge. For example, in the paper concerned with thyroid feeding (6) the following statement appears: "From this it follows that thyroid feeding increases oxidation in the blood and decreases it in the heart," etc. In another place (10) Bach and Chodat are incorrectly cited as attributing a principal rôle to catalase as an oxidizing enzyme. Kennedy and Burge (18) believe some of their experiments to indicate that the incomplete oxidation of fatty acids in diabetes is due to a deficiency in catalase inasmuch as their data show depancreatization is accompanied by a lowered catalase content of the liver and heart. This does not agree with the fact that there is increased

metabolism in diabetes. Finally dosage of some of the substances asserted by Burge to increase catalase content is so large as to make significant conclusions difficult (saccharin, 5 gm. per kilo; alcohol, 25 cc. of 45 per cent alcohol per kilo; glycine, 20 gm. per kilo, etc.).

The above criticism is offered as a preface to the description of some experiments recently made by the author which supply, it is believed, a simpler explanation of the variations in catalase content of the blood which Burge has observed and one which does not involve unproved assumptions; they do not add to knowledge concerning the function of catalase. Experiments were begun to test the validity of Burge's conclusions. To a certain extent they were confirmed but the results are too uncertain and inconstant and variations are often within such narrow limits that Burge's conclusions appear forced.

Methods.

For estimating blood catalase the procedure consisted in measuring the volume of oxygen liberated by a definite quantity of blood in a definite interval of time before and after the administration of the substance the effect of which was being investigated. The same quantity of peroxide solution (the 3 per cent solution diluted to double its volume) was used in all the determinations of any single experiment.

In the course of the experiments it was noted, as has also been remarked by other observers, that different samples of hydrogen peroxide gave varying results with the same blood. The most striking difference was found in comparing a commercial preparation with diluted perhydrol. Approximately four times as much oxygen was liberated from the perhydrol as from the ordinary commercial material, both solutions having about the same peroxide content as determined by titration with permanganate. By neutralizing the peroxide with sodium hydroxide, using neutral red, it was found possible to minimize the difference between the quantities of oxygen liberated; possibly it may be eliminated entirely. The products of several manufacturers were used in the experiments to be detailed but in the determination of any single experiment one stock solution was employed. Inasmuch as the blood in most of the experiments to

be reported was collected with the aid of potassium oxalate, it is obvious that the addition of this substance would influence the results due to the substitution of a weaker acid, oxalic, for the stronger acid present in the peroxide. The amount of oxalate in any series was constant, however, and does not invalidate the comparability of the results. In several experiments hirudine was used to prevent coagulation and diluted perhydrol substituted for the commercial peroxide of the other experiments.

An excess of hydrogen peroxide solution was placed in a bottle of about 300 cc. capacity fitted with an outlet tube through which any gas leaving the bottle was conducted to a suitable measuring vessel. A small crucible containing the blood to be tested was suspended over the peroxide and the two did not become mixed until the shaking machine[1] was started. All volumes have been reduced to standard conditions. The errors inherent in the method (changes of temperature and pressure in the course of a determination) were not in excess of 1 or 2 per cent.

EXPERIMENTAL.

The results given in Table I were obtained in a partial repetition of an experiment performed by Burge and Neill (16). These investigators found that the feeding of meat to dogs produced after 4 hours an increase of 115 per cent in the catalase content of blood drawn from the jugular vein. They state, however, that in some instances, when using dogs whose blood catalase was high, the feeding of meat produced very little or no change in the catalase of the blood of the jugular vein. In the same animals they did find an increase of from 50 to 75 per cent in the catalase content of the blood of the liver. The present experiment was performed three times. The jugular vein was exposed with the aid of cocaine and about 2 cc. of blood were drawn by means of a Record syringe which had previously been rinsed with 1 per cent potassium oxalate solution. The blood was immediately mixed with one drop of 10 per cent potassium oxalate solution and 0.5 cc. quantities were measured out with a Mohr pipette for the determinations. Several determinations were made with each sample. At the indicated intervals after feeding blood was drawn again and similar determinations were made.

[1] Made by the International Instrument Company, Cambridge, Mass.

The data obtained in the three experiments show the catalase content of blood drawn from the jugular vein is increased significantly in the second experiment only.

TABLE I.

Meat Feeding Experiments.

Experiment 1.

Weight of dog.	Weight of meat.	Oxygen liberated.			
		Before feeding.		After feeding.	
		Sample 1.	Sample 2.	60 min.	180 min.
kg.	*gm.*	*cc.*	*cc.*	*cc.*	*cc.*
5.9	208	67	60	62	63
		65	52	58	61
		66	60	60	63
		66		63	61

Experiment 2.

		Before feeding.	After feeding.		
			45 min.	120 min.	205 min.
8.0	390	33	37	40	45
		35	42	45	43
		39	38	43	
		34	40	44	

Experiment 3.

		Before feeding.	After feeding.		
			60 min.	170 min.	240 min.
7.2	270	67	57	65	68
		64	57	63	63
		66		64	68
		66		64	65

In the experiments which follow rabbits were used and blood was drawn from the ear by means of an incision sufficient to cause a ready flow of the quantity desired. 0.2 cc. was employed for the catalase determinations. Though few of Burge's published data were obtained with rabbits they are stated repeatedly to have been used and nothing is said to indicate their unsuitability for

this work. Most of Burge's analyses were made upon blood drawn from the liver, by means of a slight incision, from the portal vein, and from the jugular vein. Inasmuch as blood drawn from the jugular is shown by his data to exhibit the same general effect as blood drawn from the liver there appeared no reason why blood drawn from the ear and blood drawn from the jugular should not give comparable results. By a technique similar to that here employed Fujimoto (20) has noted an increased catalase content of rabbit blood as a result of secretin administration. Several experiments were carried out with each of the following substances: β-hydroxybutyric acid (13), saccharin (12), alanine (14), and glycine (14). The results are given in Tables II to VI.

TABLE II.

*β-Hydroxybutyric Acid Experiments.**

Rabbit No.	Before.	After.			
		35 min.	110 min.	230 min.	375 min.
	cc.	cc.	cc.	cc.	cc.
11	221	226	234	198	182
	212	213	232	201	
		30 min.	65 min.	90 min.	
12	166	179	163	165	
	153	174	183	174	
	164				

* 6 gm. per kilo of Merck's product by stomach tube.

Because the experiments here reported are very incompletely confirmatory of Burge's results, though the data are not entirely negative, it seemed desirable to seek for a more plausible explanation than Burge's hypothesis. In seeking for light an attempt was made to discover why the blood of the liver was richest in catalase. It appeared that this might be due to the presence of tissue fluid rich in catalase which found its way into the blood when the incision to collect the blood sample was made. When liver blood plasma and jugular blood plasma of the rabbit were compared, however, both showed but slight activity. Attention was next directed to the corpuscles. Senter (21) long ago showed that the catalase of the blood was confined to the cells and was

TABLE III.

*Saccharin Experiments.**

Rabbit No.	Before.		After.				
	Sample 1.	Sample 2.	30 min.	60 min.	95 min.	145 min.	160 min.
	cc.	cc.	cc.	cc.	cc.	cc.	cc.
1	202		197	215	233	215	233
	201		204	213	235	214	224
			30 min.	65 min.	130 min.		
2	126	164	149	157	157		
	127	126	143	153	149		
			45 min.	100 min.	145 min.		
3	150		150	155	163		
	145		143	146	153		
			45 min.	75 min.	105 min.	225 min.	
4	225	207	245	239	241	231	
	210	204	239	240	235		
			60 min.	110 min.			
5	138	126	132	134			
	138	126	126	125			

* 5 gm. per kilo by stomach tube.

TABLE IV.

*Alanine Experiments.**

Rabbit No.	Before.		After.		
	Sample 1.	Sample 2.	40 min.	95 min.	135 min.
	cc.	cc.	cc.	cc.	cc.
9	204		220	197	196
	196		220	204	195
	197				
			40 min.	100 min.	175 min.
10	93	91	100	95	104
	95	86	92	86	

* 5 gm. per kilo by stomach tube.

unrelated to their hemoglobin content. Could the variations in the amount of catalase be due, therefore, to variations in the number of red cells present rather than to variations in the catalase content of the red cells themselves? With this thought in mind experiments were devised to test the parallelism between variations in erythrocyte concentration and catalase content of the blood.[2] The results obtained under a variety of conditions re-

TABLE V.

*Glycine Experiments.**

Rabbit No.	Before.		After.			Remarks.
	Sample 1.	Sample 2.	30 min.	90 min.	120 min.	
	cc.	cc.	cc.	cc.	cc.	
5	126	139	149	143	137	
	129	148	149	143	137	
			50 min.	75 min.		
6	257	274	279	266		
	285	273	279	259		
			45 min.	75 min.		
7	581		560	545		Hirudine and diluted perhydrol employed.
	573		568	545		
			45 min.	90 min.		
8	656		676	623		Hirudine and diluted perhydrol employed.
	645		685	641		
	654					

* 5 gm. per kilo by stomach tube.

vealed a parallelism so striking as to lead to the conviction that observed alterations in red cell concentration are the primary cause of alterations in catalase content, and that the latter may vary with the former in a sense quite independent of the oxidative activities of individuals, tissues, or of the organism as a whole.

[2] In the paper in which Becht reports his failure to confirm Burge's thyroid and anesthesia experiments he mentions variations in the concentration of the blood as a possible source of error.

The first experiment to test this supposition consisted in a repetition of the meat feeding experiment given in Table I introducing hemoglobin estimations in addition to catalase determinations on each sample of blood taken. Usually when liver blood was collected, clotting occurred in spite of the employment of oxalate. In these cases the clots were pressed through four thicknesses of cheese-cloth in order to be able to measure out homogeneous blood samples.

TABLE VI.

Comparison of Burge's Results with Present Experiments.

Substance.	Burge's results.	Present results.
Saccharin.	56 per cent increase in 60 min.	Exp. 1 25 per cent increase in 60 min. 2 15 per cent increase in 65 min. 3 No increase. 4 14 per cent increase in 45 min. 5 No increase.
β-hydroxybutyric acid.	50 per cent increase in 90 min.	Exp. 1 No increase. 2 " "
Alanine.	21 per cent increase in 45 min.	Exp. 1 10 per cent increase in 40 min. 2 No increase.
Glycine.	25 per cent increase in 45 min.	Exp. 1 No increase. 2 " " 3 6 per cent decrease. 4 Slight increase.

All the hemoglobin estimations reported were made with a Fleischl-Miescher hemoglobinometer. The following data were obtained.

Experiment 1.—A dog weighing 7 kilos was fasted for 24 hours and the external jugular exposed with cocaine. Blood was drawn and meat fed as indicated. At the end of the experiment the animal was anesthetized and the abdominal cavity opened to obtain liver blood.

			Catalase in cc. of oxygen.	Hemoglobin.
9.30 a.m.	Jugular blood	.....	39, 37, 36	100
10.15 "	365 gm. of lean beef.			
12.15 p.m.	Jugular blood	.....	43, 43, 43	105
4.15 "	" "	.....	43, 41	107
4.30 "	Liver "	.....	50, 50	118

A parallelism between variations in catalase content and hemoglobin content is evident though the catalase appears to increase faster than the hemoglobin.

Experiment 2.—A dog of 9 kilos served for this experiment with procedure the same as in Experiment 1.

			Catalase in cc. of oxygen.	Hemoglobin.
9.15 a.m.	Jugular blood	.....	72, 73, 72, 72	86
10.30 "	" "	.....	72, 73	—
10.45 "	450 gm. of lean beef.			
12.45 p.m.	Jugular blood	.....	68, 68	84
2.45 "	" "	.....	77, 79	100
3.10 "	Liver "	.....	103, 102	117

Again the parallelism is evident but here there is a much closer correspondence in the rates at which the catalase and hemoglobin increased.

Of the five meat feeding experiments thus far reported none shows nearly so large an increase in the catalase content of the jugular blood as that shown by one dog used by Burge. It was thought that a fasting animal might give a more striking result, hence the following experiment was performed. The outcome was no more marked than in the others.

Experiment 3.—A dog weighing 8.9 kilos was deprived of food for 3½ days. The jugular vein was then exposed with cocaine and blood was drawn for the catalase and hemoglobin determinations. Meat was fed immediately and the subsequent analyses were made as indicated.

			Catalase in cc. of oxygen.	Hemoglobin.
9.30 a.m.	Jugular blood	.....	30, 31	70
9.45 "	" "	.....	35, 31	76
10.00 "	390 gm. of lean beef.			
12.15 p.m.	Jugular blood	.....	31, 33	70
2.00 "	" "	.....	31, 30	70
4.00 "	" "	.....	33, 30	70
4.20 "	Liver "	.....	46, 47	101
4.30 "	Jugular "	.....	38, 39	83

While the effect of the meat feeding upon the jugular catalase is disappointing a considerable increase in the catalase of the liver is apparent. With it goes a high hemoglobin value. The percentage increase in each is about the same. There is no obvious explanation for the slight increase in jugular catalase at the end but it is worth noting that it is also accompanied by a corresponding increase in hemoglobin.

To obtain further evidence relevant to the explanation suggested a number of experiments were carried out in which both catalase and hemoglobin determinations were made after the administration of agents which have already been reported to raise the red cell count.

A recent paper by Dale and Laidlaw (22) has shown that histamine causes a concentration of the blood as respects its formed elements. One of their experiments was repeated, therefore, with the addition that determinations of catalase were made along with the hemoglobin determinations. More marked variations were detected than in the three preceding experiments. The results are given in Experiment 4.

Experiment 4.—A cat weighing 2 kilos was anesthetized with chloroform and the anesthesia maintained with ether. Cannulas were placed in the femoral vein and carotid artery. 0.2 cc. of blood was used for the catalase determinations.

		Catalase in cc. of oxygen.	Hemoglobin.
10.00 a.m.	Carotid blood.....	150, 159	93
10.15 "	5 mg. of histamine injected.		
10.21 "	Carotid blood.....	204, 185	128

The percentage increase in hemoglobin is about 37 per cent while that of catalase is about 25 per cent.

Experiment 5.—A dog weighing 7.6 kilos was anesthetized with ether. Cannulas were placed in the femoral vein and carotid artery. This is a repetition of Experiment 4.

		Catalase in cc. of oxygen.	Hemoglobin.
11.10 a.m.	Carotid blood.....	32, 32	94
11.30 "	15 mg. of histamine injected into femoral vein.		
11.50 "	Carotid blood.....	48, 48	132
12.00 m.	" "	37, 39	113

The agreement between the percentage increases in hemoglobin and catalase is fairly good.

A third histamine experiment furnishes additional evidence.

Experiment 6.—A cat weighing 3.4 kilos was anesthetized with chloroform and the anesthesia maintained with ether. A cannula was placed in the femoral vein and blood was drawn from the jugular vein by means of a Record syringe. 0.2 cc. of blood was used for the catalase determinations.

		Catalase in cc. of oxygen.	Hemoglobin.
12.30 p.m.	Femoral blood.....	222, 220	75
1.35 "	Jugular "	220, 218	76
2.25 "	6 mg. of histamine injected into femoral vein.		
2.35 "	Jugular blood.....	292, 296	120

Here again the percentage increases in catalase and hemoglobin are in fairly good agreement.

Lamson (23) has reported increased red cell counts following the administration of adrenalin. A test was made, therefore, of the catalase content of blood after the injection of adrenalin. The experiment offered opportunity also to test the influence of histamine and of ether. Burge (7) and Burge, Neill, and Ashman (8) have reported a decreased catalase content during anesthesia but, as noted, Becht failed to confirm their results and experiments described by Lamson (23) show that there is not necessarily any change in the red cell count.

Experiment 7.—A dog weighing 8.3 kilos was employed. Its jugular vein was exposed with cocaine and blood drawn by means of a Record syringe. The animal was then etherized, blood drawn twice, and adrenalin injected by means of a Record syringe into the femoral vein. Histamine was injected later as indicated.

		Catalase in cc. of oxygen.	Hemoglobin.
9.45 a.m.	Jugular blood.....	111	97
10.00 "	" "	113, 118	98
10.15 "	Ether.		
10.20 "	Jugular blood.....	113, 114	97
10.35 "	" "	99, 108	99
11.00 "	8 mg. of adrenalin injected into femoral vein.		
11.10 "	Jugular blood.....	144, 144	117
11.25 "	" "	124, 127	114
2.05 p.m.	" "	139, 135, 139	110
2.30 "	16 mg. of histamine injected into femoral vein.		
2.38 "	Jugular blood.....	179, 167, 176	134

The parallelism between catalase and hemoglobin or red cell fluctuations is again apparent. Attention is called to the increase in catalase content obtained at the 2.05 p.m. determination. It is in discord with the comparatively low hemoglobin content of the same blood sample. A similar high catalase content, together with a correspondingly high hemoglobin value, was noted in another experiment (not reported) and it may be that under the circumstances attending such a long period of anesthesia there is an actual increase in the catalase content of the red cells. Becht's data will be found to exhibit a similar increase in catalase content when the anesthesia was long continued.

Burge has reported the catalase content of the blood of cats to be increased by fright and Lamson (23) has shown that the red cell count is increased under the same conditions. An experiment was carried out, therefore, along similar lines in which catalase and hemoglobin determinations were both made. Marked changes were not noted in either and the experiment is reported here merely to show the parallelism between catalase and hemoglobin contents.

Experiment 8.—A cat weighing about 3 kilos was removed from the animal house to a quiet room and kept there over night. The animal house is often noisy with the barking of dogs and it was thought that the preliminary period of quiet would lead to more decided results. Blood was taken from the femoral vein with the aid of cocaine for the control determinations of hemoglobin and catalase. After subjecting the animal to the barking of dogs for 5 minutes catalase and hemoglobin determinations were again made. Since the effects were very slight adrenalin was injected intravenously.

		Catalase in cc. of oxygen.	Hemoglobin.
9.45 a.m.	Femoral blood.....	193, 198	79
9.50–9.55 a.m.	Barking.		
9.55 a.m.	Femoral blood.....	212, 207	82
10.10 "	" "	196, 202	75
10.30 "	" "	204, 204	78
10.37 "	3 cc. of 0.1 per cent adrenalin solution intrafemorally.		
11.00 "	Femoral blood.....	200, 201	80
11.30 "	" "	190, 189	75
11.50 "	Liver "	209, 212	85

An explanation for the absence of any effect is not apparent but it is interesting to note that when the adrenalin did not produce the effect noted in a previous experiment on the hemoglobin content, neither was there any noteworthy variation in the catalase content.

Among other things Burge has studied the effect of sodium succinate (15) upon catalase content and because he obtained a rather marked issue in his experiment a similar one was performed.

Experiment 9.—A dog weighing 7.4 kilos was employed. Its jugular vein was exposed with the aid of cocaine and blood drawn. The abdominal cavity was next opened to obtain liver blood and for the injection of the sodium succinate in concentrated solution into the stomach and duodenum. Blood was obtained subsequently as indicated.

		Catalase in cc. of oxygen.	Hemoglobin.
10.35 a.m.	Jugular blood	67, 66	88
11.15 "	Liver "	66	88
11.35 "	60 gm. of sodium succinate..		
11.45 "	Vomiting.		
11.50 "	Jugular blood	85, 89	103
11.55 "	Purging.		
12.10 p.m.	Liver blood	105	131
12.30 "	Jugular "	95, 97	123
1.30 "	" "	100, 95	—

The amount of substance here employed was somewhat less than 10 gm. per kilo while Burge employed 14 gm. Instead of augmenting the catalase content of the blood and thereby increasing oxidation it is more likely that the osmotic effect of such large doses in this case and in many other cases reported by Burge (15, 16, 17) is so great as to cause a concentration of the blood with a consequent increase in catalase. Since the water passing into the alimentary tract must come from the abdominal circulation it is reasonable to expect the concentration to be more apparent in the liver blood than in that of the jugular or femoral veins, which may be undergoing dilution by withdrawing water from the tissues they serve.

If osmotic action furnishes the true explanation for the data reported above then such a substance as Epsom salt should be effective in increasing the catalase content of the blood. A test was made of this point.

Experiment 10.—A dog of 9.0 kilos was etherized, the abdominal cavity opened, and blood drawn from the liver by means of a slight incision. Magnesium sulfate was administered and liver blood again obtained as indicated.

		Catalase in cc. of oxygen.	Hemoglobin.
11.00 a.m.	Ether.		
11.30 "	Liver blood.....	4.8, 5.1	101
12.00 m.	15 gm. of $MgSO_4.7H_2O$ in concentrated solution injected into stomach and intestine.		
12.55 p.m.	Liver blood.....	3.8	90
2.20 "	" "	5.9, 5.9	125

The error involved when such small quantities of gas are determined by the method employed may be larger than when larger quantities of gas are being measured though it is not necessarily so. Experiment 10 while clear-cut is consequently of doubtful value by itself. Two similar experiments were performed, therefore, with dogs of higher catalase content.

Experiment 11.—A dog weighing 10.3 kilos was etherized, the jugular vein exposed, and the abdominal cavity opened. Blood was obtained as indicated before and after the introduction of magnesium sulfate in concentrated solution into the stomach and duodenum by means of a Record syringe.

		Catalase in cc. of oxygen.	Hemoglobin.
10.25 a.m.	Jugular blood.....	54, 56	114
10.35 "	Liver "	51, 51	118
10.50 "	20 gm. of $MgSO_4.7H_2O$.		
12.13 p.m.	Jugular blood.....	60, 61	131
12.20 "	Liver "	61, 59	131
1.15 "	Jugular "	62, 64	134
1.45 "	Liver "	66, 67	140
3.35 "	Jugular "	67, 69	138
3.40 "	Liver "	73, 71	151

Experiment 12.—The jugular vein of a dog weighing 12.6 kilos was exposed with cocaine and blood drawn as indicated. In this experiment the magnesium sulfate was administered through a stomach tube because when large doses were injected into the alimentary tracts of etherized animals the animals soon succumbed. The two magnesium sulfate experiments (Experiments 10 and 11) were the only successful ones out of six in which etherized animals were used.

		Catalase in cc.* of oxygen.	Hemoglobin.
1.40 p.m.	Jugular blood.....	28, 30, 31	103
1.55 "	20 gm. of $MgSO_4.7H_2O$.		
3.10 "	Jugular blood.....	37, 36	112
4.10 "	" "	36, 34	114

The data obtained in Experiments 11 and 12 accord well with those obtained in Experiment 10 and while the results are not so striking as some of the others reported nevertheless they point clearly to the existence of a close quantitative relationship existing between changes in catalase content and red cell counts.

Besides the evidence given in support of the idea that fluctuations in catalase content observed by Burge are really due to fluctuations in the red cell count attention may be called to other evidence to be found in the literature of the subject. Fujimoto (20), for example, reports that secretin calls forth an increase in the catalase content in the blood of rabbits as well as in the red cell count but he evidently regards these changes as separate phenomena.

Other data reported by Burge support the explanation offered by the present author. In experiments concerning "shock" produced in dogs by bleeding a marked decrease in catalase was observed. The cause was probably not a diminished output of catalase on the part of the liver as suggested by Burge but a dilution of the blood. In his saccharin experiments the effect may have been due to a concentration of the blood. In an attempt to repeat the saccharin experiment upon a dog there was a pronounced purgation. In Burge's experiments concerned with the effects of numerous foods (16, 17) the results may also be attributed to a concentration of the blood. The meat feeding experiments reported in this paper support such an explanation. Substances of high molecular weight or insoluble materials (raw egg white, casein, uncooked starch, dextrin, rice flour, corn-meal, wheat flour, etc.) had little or no effect upon the catalase content of the liver or jugular or portal blood. Neither do such of these substances as are soluble exert a high osmotic pressure. Substances of lower molecular weight (peptone, "aminoids," sucrose, lactose, glucose, etc.) exerted noticeable effects. Fruits such as oranges, lemons, apples, and grape fruit, which were naturally only dilute solutions when administered, exerted only very slight

positive effects. Very ripe oranges and bananas showed more marked results but the explanation is clear.

Burge has attempted to explain the specific dynamic action of food (15) on the basis of variations in catalase content but when one views the supporting data it is apparent that the changes in catalase content observed after administering glutamic acid, aspartic acid, and asparagine in doses of 5 and 14 gm. per kilo harmonize with the author's suggestion. Burge found little or no change in the case of the small doses and significant changes with the larger.

It is worth while also to call attention to the fact that the ratio between catalase content and rate of metabolism is not a constant one in some of the experiments reported. In histamine "shock," for example, it is probable that the rate of metabolism is diminished while the catalase content of the blood has been shown to be greatly increased. The same may be said of asphyxia during which Lamson (23) has shown the red cells to be present in increased number in the circulating blood. The administration of magnesium sulfate causes, as far as the writer is aware, no increase in metabolism.

SUMMARY.

Data are presented which indicate that the feeding of meat and the administration of saccharin, β-hydroxybutyric acid, alanine, and glycine are not accompanied by an increase in the catalase content of the blood to the extent reported by Burge. The increases which were noted were rather slight.

An explanation is suggested for the variations in catalase content of the blood which is supported by experimental evidence. According to this explanation fluctuations in catalase content are due to fluctuations in the number of red cells in the blood. Consequently it is simpler to regard catalase content as a function of the number of red cells than to assume a direct relation between catalase and biological oxidations.

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